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PMI 5011 regulates the ubiquitin proteasome system in skeletal muscle

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PMI 5011 REGULATES THE UBIQUITIN PROTEASOME SYSTEM IN SKELETAL MUSCLE

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LIST OF ABBREVIATIONS

Phosphatidyl-Inositol 3-Kinase (PI3K)
Type 2 Diabetes Mellitus (T2DM)
Cardiovascular Disease (CVD)
Very Low Density Lipoprotein (VLDL)
Western Blot (WB)
Low Fat Diet (LFD)
Artemisia dracunculus L (PMI 5011)
Protein Kinase B (PKB/Akt)
Muscle RING-finger protein-1 (MuRF1)
Muscle-specific ubiquitin ligase (atrogin-1/MAFbx)
Forkhead box O3 (FOXO3a)
Quantitative Real-Time PCR (qRT-PCR)
Ubiquitin Proteasome System (UPS)
Vastus lateralis Muscle (VL)
Gastrocnemius Muscle (Gastroc)
Dexamethasone (DEX)
Palmitic Acid (PA)
Insulin (INS)
Nuclear Homogenization Buffer (NHB)
Trichloroacetic Acid (TCA)
Non-Timber Forest Products (NTFP)
World Health Organization (WHO)
National Cancer Institute (NCI)
Central Drug Research Institute (CDRI)
Thin Layer Chromatography (TLC)
High Performance Liquid Chromatography (HPLC)
Size Exclusion Chromatography (SEC)
Nuclear Magnetic Resonance (NMR)
Infra Red (IR)
High Resolution Mass Spectrometry (HRMS)
Capillary Zone Electrophoresis (CZE)
High Performance Centrifugal Countercurrent Chromatography (HPCCC)
Phosphoenolpyruvate Carboxykinase (PEP-CK)
Insulin-like Growth Factor-I (IGF-I)

ABSTRACT

Insulin resistance in type 2 diabetes is associated with impaired glucose and protein metabolism in skeletal muscle. The impaired insulin signaling in skeletal muscle affects muscle mass by tilting the balance between skeletal muscle protein synthesis and degradation toward degradation, a process that is primarily regulated by the ubiquitin-proteasome system. Studies have shown that an extensively characterized ethanol extract of *Artemisia dracunculus* L (Russian Tarragon), termed PMI 5011, enhances insulin signaling in human primary skeletal muscle cells and in a rodent model of insulin resistance. The aim of this project is to determine if the effect of PMI 5011 on insulin signaling extends to regulation of ubiquitin-proteasome activity in skeletal muscle. To evaluate the effect of PMI 5011 on the ubiquitin-proteasome system, we used two *in vitro* models of insulin resistance in C2C12 myotubes and the KKA^y mouse model of insulin resistance *in vivo*. Our studies show that PMI 5011 enhances the inhibitory effect of insulin on proteasome activity and ubiquitylation in skeletal muscle *in vitro* and *in vivo*. In addition, PMI 5011 inhibits non-proteasomal protein degradation *in vivo*, indicating that PMI 5011 is a potent inhibitor of skeletal muscle protein degradation. PMI 5011 also regulates the expression of Atrogin-1 and MuRF-1, muscle-specific ubiquitin ligases that are required for ubiquitin-dependent protein degradation in skeletal muscle. Both Atrogin-1 and MuRF-1 gene and protein expression is elevated with impaired insulin signaling and our studies show that PMI 5011 reduces the expression of these ligases while enhancing Akt phosphorylation. In summary, these studies demonstrate that PMI 5011 regulates the ubiquitin-proteasome in insulin resistant states *in vitro* and *in vivo*. PMI 5011 may therefore be a therapeutic target for enhancing insulin sensitivity leading to conservation of muscle mass in type 2 diabetes.

CHAPTER 1. INTRODUCTION

1.1 Discovery and Use of Medicinal Plants from NTFPs and PMI 5011

Natural drugs from traditional medicines are gaining popularity due to fewer side effects, reduced cost, and increased patient use (Phillipson, 2001). NTFPs are those plants or plant parts found within the forest that are not considered for use as timber. They include mushrooms, edible fruits and berries, leaves, roots, and bark utilized for herbal medicines, as well as wood for carving, twigs for decorative baskets or wreaths and many others. From these NTFPs, herbal medicines derived from forest products constitute the highest valued segment of the NTFP industry (Chamberlain and Hammett, 1998). It is also thought that NTFPs contribute to sustainable forest management, conservation and to economic and development objectives (Panayotou and Ashton, 1992). According to the World Health Organization (WHO) traditional and herbal medicines are used throughout the world and constitute 70-95% of the primary therapeutic agents in developing countries. They estimate that the global market for herbal medicines in U.S. currency annually as of 2008 is $83 billion with an expected exponential increase (WHO, 2011). There is evidence in the fossil record that humans have been utilizing plants for medicinal purposes as far back as 60,000 years ago (Solecki, 1975). In the United States, the earliest recorded use of herbal medicines by Native Americans occurred in the 1600's. These herbal medicines were passed on to American settlers who also used the medicinal remedies brought from their own countries. These herbal medicines were used until the early 1900's when the synthesis of natural product substitutes first began. At that time, there was a major shift from natural plant remedies to synthetic drugs and over the next

century the Federal government became involved by enacting several Amendments and Acts to regulate both herbal medicines and synthetic drugs used in America (Rates, 2001). Since the 1990's there has be a resurgence of interest in naturally derived medicines. Of the top 20 prescriptions in 1996, six were natural products (Phillipson, 2001). During the 1990's, medical research on such drugs as taxol from the Pacific yew, etoposide from the American mayapple and artemisinin from annual wormwood helped increase an interest and demand for botanical sources of medicinal drugs thereby increasing the demand for non-timber forestry products (Chamberlain and Hammett, 1998). "Natural products have contributed nearly half of all small molecules approved in the past decade" (Patwardhan and Mashelkar, 2009). The number of higher plant species on this planet is listed at 500,000 and of these, 6% has been screened for biological activity and only 15% has been phytochemically tested (Verpoorte, R. 2000). In biomedical research, the goal of using medicinal plants as a source of therapeutic agents is to target the isolation of the bioactive compounds for synthesis and direct use as drugs and to use compounds as pharmacologic tools or as an herbal remedy. The process usually starts with previous knowledge of a particular plant that has been recorded in history followed by biological and toxicological assays for activities of interest. The process may also include random collection and selection of plant materials followed by phytochemical screening approaches for selection of specific secondary plant metabolites such as flavonoids, alkaloids, etc. (Farnsworth, 1966). This selection is then followed by biological and toxicological assay assessment, most commonly by *in vitro* assays followed by *in vivo* assessment (Fabricant and Farnsworth, 2001). This process then leads to further fractionation and isolation of pure compounds through numerous extraction techniques

including cold extraction, hot percolation, supercritical fluid extraction or soxhlet extraction followed by chemical characterization with chromatography, (TLC, HPLC, HRMS, HPCC, and SEC) NMR, CZE, X-ray analysis or Mass Spectrometry (HRMS) that leads to synthesis of the active components (Gurib-Fakin, 2006 and Borris, 1996). This process is summarized below in Figure 1.1.

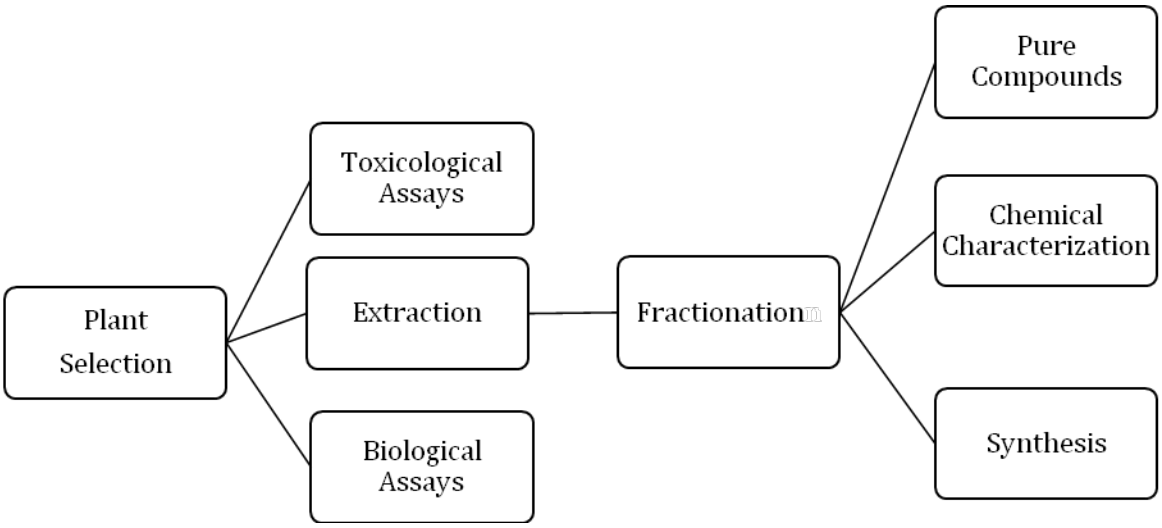


Figure 1.1. Method for biological and toxicological assay guided active compound selection.

Adapted from Rates (2001).

In this way, drugs have been discovered and are now commonly used. To date, some hundred or so drugs have been developed from medicinal plants. An abbreviated list of some examples of these drugs discovered from plants is listed in Table 1.

Table 1. Drugs derived from plant sources.

Drug	Action	Plant Source
Acetyldigoxin	Cardiotonic	*Digitalis lanatat* Ehrh.
Anisodine	Anticholinergic	*Anisodus tanguticus* Pascher
Atropine	Anticholinergic	*Atropa belladonna* L.
Berberine	Bacillary dysentery	*Berberis vulgaris* L.
Caffeine	CNS stimulant	*Camellia sinensis* (L.) Kuntz
Camphor*	Antimicrobial	*Cinnamonum camphora* L.
Camptothecin*	Anticancer	*Camptotheca acuminata* Decne.
Codeine	Analgesic; antitussive	*Papaver somniferum* L.
Curcumin	Choleretic	*Curcuma longa* L.
Digitalin, Digitoxin	Cardiotonic	*Digitalis purpurea* L.
Ephedrine	Sympathomimetic	*Ephedra sinica* Stapf.
Etoposide	Antitumour agent	*Podophyllum peltatum* L.
Gossypol	Male Contraceptive	*Gossypium* spp.
Kawain	Tranquilizer	*Piper methysicum* Forst. f.
Khellin	Bronchodilator	*Ammi visnaga* (L.) Lamk.
Metformin	Antidiabetic	*Galega officinalis* L.
Morphine	Analgesic	*Papaver somniferum* L.
Physostigmine	Cholinesterase inhibitor	*Physostigma venenosum* Balf.
Pseudoephedrine	Sympathomimetic	*Ephedra sinica* Stapf.
Quinine	Antimalarial	*Cinchona ledgeriana* Moens.
Rhomitoxin	Antihypertensive	*Rhododendron molle* G. Don
Salicin	Analgesic; Anti-inflammatory	*Salix alba* L.
Santonin	Ascaricide	*Artemesia maritima* L.
Scopolamine	Sedative	*Datura metel* L.
Taxol*	Anticancer	*Taxus brevifolia* Nutt.
Thymol*	Antiseptic	*Thymus vulgaris* L.
Tubocurarine	Muscle Relaxant	*Chondodendron tomentosum* R. & P.
Vinblastine*	Anticancer	*Catharanthus roseus* (L.) G. Don
Xanthotoxin	Leukoderma; vitiligo	*Ammi majus* L.

Data adapted from Fabricant (2001) * Drugs discovered in random selection by the NCI and CDRI

1.2 Diabetes Prevalence, Causes, and Treatment

Diabetes mellitus is a metabolic disease characterized by high blood glucose.

There are three main types of diabetes: type 1 diabetes that results from the body's

inability to produce insulin and is considered insulin-dependent diabetes; type 2 diabetes that results from the development of resistance to the body's own insulin supply, often caused by obesity; and gestational diabetes that occurs during pregnancy that can proceed the development of type 2 diabetes later in life (Expert Committee Report on the Diagnosis and Classification of Diabetes Mellitus, 2002). The high blood glucose associated with type 2 diabetes is a result of the body's impaired response by peripheral tissues to insulin. The reduction in the cellular uptake of glucose due to this insulin resistance leads to the high blood glucose levels that are characteristic of type 2 diabetes. This decreased cellular response to insulin or perturbation of the insulin signaling pathways are associated with a number of pathological states (Taylor, 1992), including cardiovascular diseases such as atherosclerosis and hypertension (Savage, *et al.*, 2005; Reaven, 2003). Diabetes is the seventh leading cause of death in the United States (Wild, *et al,* 2004). According to the Centers for Disease Control, diabetes affects over 26 million Americans with 79 million having pre-diabetes defined as having fasting blood glucose levels of 110 to 125 mg/dL (WHO criteria) or 100 to 125 mg/dL (ADA criteria). According to the American Heart Association (American Heart Association, 2008), diabetes contributes to about 225,000 U.S. deaths per year.

1.3 Insulin Resistance and Muscle Atrophy

In states of insulin resistance such as type 2 diabetes, both glucose and protein metabolism are altered (Park, 2009). In the body, skeletal muscle is both the primary storage site for proteins and the major site of glucose disposal. Approximately 80% of insulin-stimulated glucose disposal occurs in skeletal muscle. Insulin plays a major role

in regulating muscle protein metabolism, and its action in skeletal muscle helps to maintain the balance between protein synthesis and degradation. Insulin stimulates protein synthesis by activating transcription and by increasing tissue and cellular capacity for protein synthesis (Proud, 2006). At the same time, insulin also inhibits protein degradation by downregulating proteasome activity and the transcription of enzymes in the ubiquitin system (Sacheck, *et al.,* 2004). On the other hand, insulin resistance leads to an imbalance between protein synthesis and breakdown in skeletal muscle, resulting in structural and functional modifications of skeletal muscle proteins and the loss of muscle mass. Recently, specific actions of insulin on various muscle proteins was shown to play a major role in regulating muscle protein metabolism, and the dysregulation of insulin action is thought to contribute to muscle wasting, also referred to as muscle atrophy (Guillet and Boirie, 2005). Studies show that skeletal muscle atrophy occurs in response to a number of catabolic conditions, such as fasting and several diseases such as AIDS, sepsis, cancer, Cushing's syndrome, and diabetes mellitus (Sandri, *et al.*, 2004, Glass, 2003, Wang, *et al.,* 2006 and Lecker, *et al*., 2004). In older, sedentary individuals, especially women, there is a significant loss of muscle mass due to skeletal muscle protein breakdown caused by the insulin resistance of type 2 diabetes (Wang, *et al.,* 2006; Price, *et al.,* 1996; Mitch, *et al.,* 1999). This insulin resistance leads to a decrease in lean body mass and physical disabilities, including sarcopenia, that are associated with morbidity and mortality (Wang, *et al.,* 2006).

A. Dexamethasone-Induced Insulin Resistance

The hormone cortisol is secreted from the adrenal cortex in response to inflammation, pain, infection and stress (Newton, 2000). It is called a glucocorticoid because it is essential for long-term blood glucose maintenance (Champe and Harvey, 1994) and is a counter-regulatory hormone due to its opposing actions on insulin-mediated metabolic functions. Cortisol opposes insulin's actions by decreasing glucose uptake into cells and decreasing insulin secretion by pancreatic β-cells. Cortisol affects peripheral glucose metabolism by interrupting insulin-stimulated glucose uptake, including insulin receptor binding (Olefsky *et al.*, 1975 and Buren *et al.*, 2002). These actions can lead to hyperinsulinemia, hyperglycemia and insulin resistance (Rizza *et al.,* 1982; Andrew and Walker, 1999: and Newton 2000). Glucocorticoids such as dexamethasone decrease the rate of protein synthesis and increase the rate of protein breakdown causing atrophy of skeletal muscle that is under the regulation of insulin and insulin-like growth factor-I (IGF-I) (Goldberg, *et al.*, 1980 and Lofberg *et al.*, 2002). Therefore, in insulin resistant states such as those induced by glucocorticoids, muscle atrophy is upregulated. The glucocorticoid-induced protein degradation is due to activation of the ubiquitin-proteasome system (Menconi, *et al.,* 2008; Tiao, *et al.,* 1996; Schakman, *et al.,* 2008). Administration of the synthetic glucocorticoid, dexamethasone (DEX), results in increased expression of the two muscle specific E3 ubiquitin ligases Atrogin-1 and MuRF-1, (Menconi, *et al.,* 2008; Sandri, *et al.,* 2004; Stitt, *et al.,* 2004) whose activity leads to muscle atrophy. The Dex-induced increase in Atrogin-1 and MuRF-1 expression is dependent upon ligand binding to the glucocorticoid receptor (Zhao, *et al.,* 2009) leading to the activation of the transcription factors FoxO1and

FoxO3a that in turn upregulate the expression of Atrogin-1 (Sandri, *et al.,* 2004; Skurk, *et al.,* 2004) and MuRF-1 (Waddell, *et al.,* 2004).

B. Free Fatty Acid-Induced Model of Insulin Resistance *In vitro.*

Free Fatty Acid (FFA) deposition that occurs in insulin-sensitive tissues such as the liver, pancreas and skeletal muscle (Jensen, 2006) causes systemic insulin resistance. In obesity-related type 2 diabetes there is an associated increase in circulating plasma free fatty acid (FFA) levels (Boden, 2006). First described by *Randle in 1963*, the contribution of circulating plasma FFAs to the insulin resistance associated with type 2 diabetes is now well established. Their work carried out in rat heart and diaphragm muscles originally focused on the effect of FFA on glycolysis (Randle, 1963). Later efforts demonstrated that FFAs inhibit insulin's action by inhibiting intracellular pathways of glucose metabolism at the level of glucose transport and PI3K signaling pathway (Dresner*,* 1999). FFA-induced insulin resistance occurs predominately in skeletal muscle to a greater extent than in either the liver or endothelial cells (Boden, 2006). This can be observed within 2-4 hours of an increase in plasma FFA. A person with a rise in plasma FFA from 400 to 800 pM after a fatty meal displays a reduction in the effect of insulin by 50% (Boden, 2007). A gradual increase in triglycerides, diacylglycerol (DAG) and the long chain acyl-CoA within muscle cells caused by elevated FFAs is thought to be the underlying cause of insulin resistance in skeletal muscle. This accumulation leads to inhibition of PI3K activation of Akt, thereby inhibiting insulin signaling on two levels. Chavez *et al.*, 2005, showed that in C2C12 myotubes, free fatty acids (FFA) inhibited the insulin stimulation of Akt/PKB signaling

that regulates glucose uptake in addition to protein synthesis and the transcriptional regulation of both Atrogin-1 and MuRF-1 atrogenes involved in skeletal muscle atrophy.

1.4 Muscle Protein Degradation and the Ubiquitin-Proteasome Pathway

The skeletal muscle atrophy associated with type 2 diabetes and insulin resistance is caused in part, by ubiquitin-mediated proteolysis and the expression of two genes encoding ubiquitin ligases, MAFbx/Atrogin-1 and MuRF1, that is increased in muscle atrophy (Glass, 2003) and contribute most significantly to muscle atrophy (Lagirand-Cantaloube, *et al.*, 2009). The ubiquitin-proteolytic pathway catalyzes the breakdown of polypeptides and proteins in a process where multiple ubiquitin moieties are covalently conjugated to amino groups on the targeted proteins, marking them for degradation by the 26S proteasome. This process involves three sequential enzymatic reactions performed by three types of enzymes: E1, E2, and E3 (Medina, *et al.,* 1995 and Herrmann, *et al.,* 2007). Ubiquitin is first activated by ubiquitin-activating enzyme E1, in an ATP-dependent manner. Next, the ubiquitin molecule is then passed on to the second enzyme of the complex, E2 ubiquitin-conjugating enzyme. Next, it is recognized by the final enzyme, E3, the ubiquitin protein ligase that binds the target substrate and labels it with ubiquitin. The process can be repeated until a short chain is formed, with three or more ubiquitin molecules targeting proteins for degradation at the proteasome (Wing, 2005). The expression of the ubiquitin ligases Atrogin-1 and MuRF-1 is increased in all forms of catabolic diseases that lead to muscle loss (Foletta, 2011). One potential proteolytic trigger of skeletal muscle protein breakdown is a decrease in the response to insulin or Insulin-like growth factor 1 (IGF-1) (Wang, *et al.,* 2006). Impaired insulin signaling via

the PI3K/AKT signaling cascade causes an imbalance between protein synthesis and degradation that favors degradation of proteins in skeletal muscle (McKinnell and Rudnicki, 2004). Loss of skeletal muscle mass and function is the result of prolonged and accelerated protein degradation associated with insulin resistance (Mitch and Goldberg, 1996). Insulin binds to the insulin receptor tyrosine kinase and initiates the phosphatidylinositol 3-kinase/Akt (PI3K/AKT) signaling pathway that plays a major role in metabolism, cell growth and proliferation (Glass, 2003 and 2010). Insulin-dependent activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/AKT) leads to a phosphorylation cascade affecting several downstream targets, including FoxO3a gene expression (Proud, 2006, Schakman, *et al.*, 2008, Sandri, *et al.*, 2004, Zhao, *et al.*, 2007). Increased FoxO3a protein expression activates transcription of Atrogin-1 and MuRF1 (Schakman, *et al.*, 2008). The IGF-1/PI3K/Akt pathway prevents the expression of these two ubiquitin ligases by inhibiting FoxO transcription factors (Stitt, *et al.*, 2004). Phosphorylation of FoxO proteins leads to their exclusion from the nucleus and inhibition of FoxO3a-mediated MuRF1 and Atrogin-1 gene expression, thereby blocking ubiquitin-mediated muscle atrophy (Glass, 2010).

1.5 Treament of Insulin Resistance and Type 2 Diabetes

The American Diabetes Association makes the following recommends in their Standards of Medical Care in Diabetes—2012 issue of Diabetes Care;

1. Glucose monitoring daily
2. A1C testing annually
3. Lifestyle interventions - Diet and Exercise
4. Medical Nutrition Therapy
5. Pharmacological Interventions

a) Insulin-Type I diabetes and in extreme cases of Type II and gestational diabetes.
b) Biguanide Class - i.e. Metformin**
c) Sulfonylureas
d) Meglitinides
e) Thiazolidinediones (Glitazone)
f) α-glucoside inhibitors
g) GLP-1 receptor agonists (incretin mimatics)
h) DPP-4 inhibitors (incretin enhancers)
i) Bile acid sequestrants
j) Dopamine-2 agonist

6. Bariatric Surgery - in extreme cases.

**=Plant-based Diabetic Drugs.

Many of these interventions such as lifestyle changes in diet and exercise are not easily achieved nor maintained over extended periods (Curioni and Lourenco, 2005 and Tate, *et al*., 2007). In addition, dangerous surgeries such as bariatric surgery should be avoided at all costs due to complications and expense. Lastly, pharmacological interventions have provided a powerful tool in combating the complications of type 2 diabetes. Recently, there has been a renewed interest in natural products in the treatment of several diseases including type 2 diabetes.

1.6 *Artemisia dracunculus* L. (PMI 5011), Metformin and Diabetes.

Many plants have been described in both clinical research and traditional medicine to have antidiabetic properties including improved glucose uptake and reduced insulin resistance. Medicinal herbs have been used for antidiabetic properties and contain herbal extracts that restore the function of pancreatic tissues and cause increased insulin output by the functioning beta cells, while other ingredients enhance the microcirculation, and increase the availability of insulin and facilitate metabolism in insulin-dependent processes (Jia, 2003). Some examples of the most commonly used plant based botanical products that have been studied and used for their antidiabetic

properties are bitter melon (*Momordica charantia*), fenugreek (*Trigonella foenum-graecum*), gurmar (*Gymnema sylvestre*), ivy gourd (*Coccinia indica*), prickly pear cactus (*Optunia streptacantha*), ginseng (*Panax, sp.*), aloe vera (*Aloe barbadensis*), garlic (*Allium sativum*) and Russian tarragon (*Artemisia dracunculus*) (reviewed in Cefalu, *et al.*, 2008).

Many drugs in the past were originally derived from plants. One drug of particular interest is metformin. Metformin was originally discovered from a plant source *Galega officinalis* commonly known as goat's rue or French lilac. Metformin is one of the most commonly prescribed drugs in the treatment of type 2 diabetes and is often the first line of defense chosen by physicians. Its original use can be traced to medieval times, when it was used to relieve the frequent urination associated with diabetes mellitus (Witters, 2001). Metformin not only lowers blood glucose levels but it also inhibits adipose tissue lipolysis, reduces circulating free fatty acids, and diminishes very low density lipoprotein (VLDL) production (Wood, *et al*., 1996). The glucose-lowering effect of metformin is caused by a combination of several distinct activities in various organs and tissues. Among the known actions of metformin are an improvement of insulin sensitivity in both the muscle and liver, a decrease in hepatic glucose production from gluconeogenesis, an increase in peripheral glucose utilization through stimulation of insulin-mediated muscle glucose uptake and glycogen synthesis as well as positive effects on insulin receptor expression and tyrosine kinase activity (Cusi, *et al*., 1996 and Stepensky, *et al*., 2002). Metformin is a clear example of the impact of plant-based drug discovery. A renewed interest from the pharmaceutical industry and the general public in the use of plants as medicinal therapies, as sources of new lead molecules and as conventional and

complementary therapies has led to a recent and significant increase in research to discover new botanical compounds for future drug development (Li, *et al.*, 2004).

This dissertation focuses on *Artemisia dracunculus* L, the plant commonly referred to as Russian tarragon. It is a perennial herb belonging to the Asteraceae family. There are over 1500 species in the plant genus *Artemisia*, and it has been utilized for both herbal remedies as well as conventional drugs. In addition to *A. dracunculus,* some examples of the medicinal applications of *Artemisia* species include *A. annua* as a malaria treatment, and three other *Artemisia* species used to treat diabetes are; *A. herba-alba* as a tea, *A. pallens* ethanolic extracts, and *A. santonicum* that are being used in traditional medicine throughout the world (Ribnicky, *et al*., 2006). The ethanolic extract of *Artemisia dracunculus* is termed PMI 5011. PMI 5011 was originally identified from a screening of extracts for hypoglycemic activity in diabetic mice and was identified as the most promising candidate for the development of a nutritional supplement for the treatment of type 2 diabetes (Cefalu, *et al.*, 2008). Studies have shown that PMI 5011 decreases circulating blood glucose and improves insulin levels in both *in vivo* and *in vitro* models of type 2 diabetes (Ribnicky, 2006 & 2009, Wang, *et al*., 2008 & 2010, Kirk, *et al*., 2008, Zuberi, 2008, and Obanda, *et al*., 2012). The extract enhances insulin stimulated glucose uptake and increases the levels of insulin receptor substrate-2 (IRS-2) in skeletal muscle cells of obese rats (Ribnicky, *et al*., 2006). The active compounds in the extract are polyphenols that are members of the sesquiterpene lactone and flavonoid groups, of which the *Artemisia* family is well known (Ribnicky, *et al*., 2006). Current data further suggests that PMI-5011 may improve carbohydrate metabolism by enhancing

the molecular actions of insulin in skeletal muscle (Ribnicky, *et al*., 2006, Cefalu, *et al*., 2008, Wang, *et al*., 2008 & 2010 and Obanda, *et al*., 2012).

1.7 Polyphenols, Carbohydrate Metabolism, and Diabetes

Polyphenols are a structural class of organic chemicals mainly found in nature that are characterized by their multiple phenol structural groups. Plants mainly synthesize polyphenols in addition to certain fungi and a few animals. Polyphenols are protective chemicals in plants and provide a defense against predators/infections, defense against sunlight damage and chemical oxidation, and they provide coloration (Guyente, 2011). The color of many fruits and vegetables, such as blueberries, eggplants, grapes and apples comes from polyphenols. The number of phenol groups and characteristics of these structures give them their unique physical, chemical, and biological (metabolic, toxic, therapeutic, etc.) properties. The four main classes are the phenolic acids, flavonoids, lignans and stilbenes. Polyphenols are mainly known for their antioxidant properties and have been studied in implications as preventatives for oxidative-related diseases such as hypertension, diabetes and cardiovascular disease (CVD) (Manach, *et al.,* 2004). The structural diversity of the polyphenols provides differences in biological activities and bioavailability (Scalbert and Williamson, 2000 and Manach, *et al.,* 2004). Phenolic acids containing only one phenol ring are the most basic of the polyphenols and the most commonly found in nature. The flavonoids contain 2 aromatic rings linked together by oxygenated heterocycle consisting of 3 carbon atoms and are classified further into 6 subclasses: flavanols, flavones, flavanones, isoflavones, anthocyanidines and flavanols (catechins and proanthocyanidins). Lignans are dimeric compounds that

contain two phenylpropane units connected by a central carbon of their side chains. Lastly, the stilbenes are isomeric hydrocarbons and are the least common. Resveratrol is the most popular of stilbenes studied (Manach, *et al,* 2004).

Chalcones are flavonoids lacking a heterocyclic C ring (Calliste *et al*., 2001) and are α-β-unsaturated ketones that have been shown to have anti-inflammatory, anticancer, antiviral, antiprotozoal, antibacterial, antifungal and insecticidal activities as well as having enzyme-inhibitory activities (Dimmock, *et al.*, 1999). The anti-hyperglycemic active components found in PMI 5011 are 6-demethoxycapillarisin and 2', 4' dihydroxy-4-methoxydihydrochalcone (Govorko, *et al.*, 2007). 2', 4' dihydroxy-4-methoxydihydrochalcone (DMC) is a chalcone and plant stress compound described as a *de novo* metabolite of stressed plants (Carlson and Dolphin, 1981).

1.8 Medicinal Plants and the Ubiquitin Proteasome

The ubiquitin-proteasome pathway is responsible for energy-dependent protein degradation and is involved in many cellular processes such as cell-cycle regulation, transcription, proliferation, apoptosis and angiogenesis. Studies show that cancer cells are more susceptible to proteasome inhibition than normal cells (Yang, *et al.*, 2008). Therefore, proteasome inhibitors have been targeted as anticancer drugs targets. There are several types of proteasome inhibitors including peptide aldehydes (MG132, MG115, and LLnL), peptide boronate, peptide vinyl sulfone, peptide epoxyketone, and naturally derived sources (Screen, *et al.,* 2010). To date, the peptide aldehydes are the most understood and utilized proteasome inhibitors. One of the first proteasomal inhibitors was isolated from a natural compound in the 1990's called lactacystin and it was

originally discovered in microbial Streptomyces. Other natural product proteasomal inhibitors include the polyphenol EGCG derived from green tea (*Camellia sinensis*), several polyphenol flavonoids including resveratrol, apigenin, and quercetin from grape extracts, polyphenol isoflavones called genistein from soy, polyphenol flavonoids from turmeric called curcumin, quione methide tripterene called celastrol from the *Tripterygium wilfordii* and a methyl ester of celastrol from *Celastraceae* family called pristimerin. All have been found to block chymotrypsin-like activity of the proteasome (Yang, *et al.*, 2008; Chen, *et al.*, 2011). Possibly the most studied natural product proteasome inhibitor of late is the green tea polyphenol known as (-)-epigallocatechin gallate [(-)-EGCG] that has been shown to inhibit proteasomal activity in cancer cells. However, much like other natural products it has been found unstable under physiological conditions. Dou *et al.* developed a peracetate-protected or pro-drug form of (-) EGCG termed Pro-EGCG that increases the stability and bioavailability, thereby improving the proteasome-inhibitory and anticancer activities (Dou *et al., 2008)*. Recently, Alamadari, *et al.* reported on the polyphenol resveratrol (3,5,4'-trihydroxystilbene) and its inhibition of dexamethasone-induced expression of the two E3 ubiquitin ligases Atrogin-1 and MuRF-1. Their studies *in vitro* in murine L6 myotubes showed that glucocorticoid induced atrophy were blocked with treatment of resveratrol in a SIRT1 dependent manner (Alamadari, *et al.*, 2012). Other studies on resveratrol have provided evidence for the muscle sparing effects provided by this compound in diseases that are associated with muscle wasting such as diabetes, muscular dystrophy, muscle disuse and cancer (Alamadari, *et al.*, 2012). Natural compounds such as resveratrol and

those active components of the extract of *Artemesia dracunculus* have important clinical implications for therapeutic uses for muscle atrophy associated with disease.

CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1 PMI 5011 Extract Preparation

The PMI 5011 botanical extract from *Artemisia dracunculus* L. was provided by the Botanical Centers at Rutgers University and Pennington Biomedical Research Center. Detailed information about quality control, preparation, and biochemical characterization of PMI 5011 has been previously reported (Ribnicky, *et al.,* 2005). In brief, the seeds of *Artemesia dracunculus* L. were purchased from Sheffield's Seed Co. Inc. (Locke, NY). Plants were grown hydroponically in greenhouses under uniform and strictly controlled conditions, thereby standardizing the plants for their phytochemical content. Total plants were harvested above the root mass, frozen and stored at -20º C prior to extraction through ethanolic preparations. The extract has been extensively characterized through the isolation of active components by activity-guided fractionation using *in vitro* bioassays followed by confirmation *in vivo* (Ribnicky, *et al.,* 2005). Purification, isolation and identification were achieved with high performance liquid chromatography (HPLC) analysis and liquid chromatography-mass spectrometry (LCMS) analysis. For further detail on complete experimental procedures, please refer to Ribnicky, *et al.,* 2005.

2.2 Cell Culture

C2C12 cells are derived from myoblast cells isolated from the thigh muscle of two-month-old C3H mice (Yaffe and Saxel, 1977). C2C12 cells are a diploid subclone selected for their ability to differentiate rapidly and produce extensive contracting myotubes that express characteristic muscle proteins (Blau, *et al.,* 1985). The C2C12

cells are differentiated into myotubes by transferring the cells from 10% fetal bovine serum to 2% horse serum once they have reached confluence. These cells are commonly used as a tool to study skeletal muscle protein and gene expression, differentiation of myoblasts and the mechanistic pathways related to skeletal muscle biology. C2C12 murine myoblasts were obtained from ATCC and grown in complete growth medium of Dulbecco's Modified Eagle's Medium, 1g/L glucose without sodium pyruvate (DMEM-Cellgro), but with L-glutamine, 10% fetal bovine serum (HyClone) and 1% Penicillin-Streptomycin (MP Biomedicals). Cells are grown to sub-confluence at 37°C at 5% CO_2. To induce myotubes formation, cells are allowed to become confluent and then supplemented with 2% horse serum (HyClone) in place of fetal bovine serum and grown at 37°C at 5% CO_2. C2C12 myotubes 6-9 days post-induction were used for all experiments *in vitro*. C2C12 myotubes were preincubated with PMI 5011 (10 μg/ml) for 16 hours prior to the addition of wortmannin (200 nM) for PI3K signaling assays. To induce insulin resistance, differentiated myotubes were treated with the glucocorticoid dexamethasone at 1μM concentration for 24 hours. Cells were also incubated in the absence or presence of the botanical extract PMI 5011 at 10μg/ml overnight. As another model of insulin resistance *in vitro*, differentiated C2C12 myotubes were treated with the free fatty acid palmitic acid at 200 μM overnight with and without 10μg/ml of PMI 5011. Both insulin resistance models are discussed in detail below.

2.3 Induction of Insulin Resistance

1. Dexamethasone Treatment. C2C12 myotubes were initially incubated in the absence or presence of dexamethasone (1 μM) (Sigma Aldrich, St. Louis, MO) for 24 hours.

Subsequent experiments were carried out in the absence or presence of dexamethasone and PMI 5011 (10 μg/ml) for 24 hours. When added, PMI 5011 was present for 4 hours prior to the addition of dexamethasone. Twenty four hours after adding dexamethasone, the cells were harvested for isolation of RNA and whole cell extracts.

2. Free Fatty Acid Treatment. Palmitic acid (Sigma Aldrich, St. Louis, MO) was diluted in ethanol at 100 mM and further diluted to a 6 mM working solution in 2% fatty acid free Bovine Serum Albumin (BSA) in DMEM. The 6 mM solution was briefly sonicated and incubated for 20 minutes at 55° C until a clear solution was observed. The palmitic acid was then diluted to the desired final concentration and sterile filtered. C2C12 myotubes were incubated in the absence or presence of palmitic acid (200 μM) and PMI 5011 (10 μg/ml) for 16 hours in DMEM, 10% FBS. Thereafter, the media was exchanged for DMEM containing 0.3% fatty acid free BSA in the presence or absence of palmitic acid and PMI 5011for 6 hours prior to insulin stimulation (100 nM insulin). Two hours after adding insulin, the cells were harvested for isolation of RNA and whole cell extracts.

2.4 Preparation of Whole Cell Extracts

Skeletal muscle tissue lysates were prepared by dissecting the muscle free of adipose tissue and homogenizing in 25 mM HEPES, pH 7.4, 1% Nonidet P-40 (NP-40), 137 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml leupeptin using a PRO 200 homogenizer (PRO Scientific, Inc., Oxford, CT). The samples were centrifuged at 14,000×*g* for 20 min at 4°C. Whole cell extracts were harvested from the C2C12 myotubes in a lysis buffer containing 50 mM Tris-Cl, pH 7.4 with 150 mM NaCl,

1 mM EDTA, 1% Igepal, 0.5% Na-deoxycholate, 0.1% SDS, 10 mM N-EM, and protease inhibitors (1 μM PMSF, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin) and lysed via sonication. In each case, protein concentrations were determined using a BCA assay (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. The tissue supernatants (50 μg) and C2C12 whole cell extracts (50 μg) were resolved by SDS-PAGE and subjected to immunoblotting using chemiluminescence detection (Thermo Fisher Scientific, Rockford, IL) and quantified.

2.5 Gel Electrophoresis and Immunoblotting

Proteins were separated in 10% polyacrylamide (acrylamide from National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (Laemmli, 1970) and then transferred to nitrocellulose membrane at 25 volts overnight at 4ºC in 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were blocked in 4% fat-free milk for 1 hour at room temperature before incubation in primary antibody for 2-4 hours at room temperature at concentrations recommended by manufacturer. Protein expression for Atrogin-1(ECM Biosciences), MuRF-1 (AbCam), ubiquitin (BD Pharmingen), phosphorylated (S473) AKT (Cell Signaling), total AKT (Cell Signaling), eIF3F (Bethyl), Foxo3a (Bethyl), phosphorylated (S253) Foxo3a (Millipore), mTOR and phosphorylated (S2448) mTOR (Cell Signaling) and β-Actin (Bethyl) were determined and compared to total protein content. Membranes were then exposed to the appropriate secondary antibody at the concentration recommended by the manufacturer for 1 hour at room temperature. Proteins were visualized with enhanced chemiluminescence (Pierce).

2.6 RNA Isolation and Analysis

Total RNA was purified from the C2C12 cells using TriReagent (Molecular Research Center) according to the manufacturer's instructions. Total RNA was purified from the skeletal muscle tissue using an RNeasy Fibrous Tissue Minikit (Qiagen) according to the manufacturer's directions. In each case, RNA (1 μg) was reverse transcribed using Multiscribe Reverse Transcriptase (Applied Biosystems) with random primers at 37^0C for 2 hours. Real-time PCR was performed with TaqMan two-step chemistry with TaqMan primer/probe pairs using the 7900 Real-Time PCR system (Applied Biosystems) and universal cycling conditions (50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds and 60 °C for 1 minute; followed by 95°C for 15 seconds, 60 °C for 15 seconds and 95°C for 15 seconds. Relative gene expression of Atrogin-1, MuRF-1, 6S proteasome subunits PSMA5 and PSMB3, Ubiquitin B, Ubiquitin C and Ubiquitin A52 were measured and compared to an endogenous housekeeping gene (cyclophilin B). Samples were measured for quantitation of total RNA content by the NanoDrop, UV-Vis instrument (Thermo Scientific).

2.7 Rodent Care

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Pennington Biomedical Research Center in accordance with ALAC guidelines for the use of experimental animals. Six-week-old male KK-Ay mice (n=16) purchased from the Jackson Laboratory (Bar Harbor, ME, USA) were single housed in specific pathogen-free animal rooms maintained at 25°C with a 12-h light–dark

cycle (8 a.m.–8 p.m.). The mice were maintained on a defined low-fat diet containing 16.4 kcal% protein, 10.5 kcal% fat, and 71.3 kcal% carbohydrate (D12329; Research Diets, Inc., New Brunswick, NJ, USA). At 10 weeks of age, the mice were randomly divided into a control group (N=8) and a PMI 5011-treated group (5011; n=8). The 5011 treatment group was fed *ad libitum* the same defined low-fat diet containing 1% (w/w) of PMI 5011, whereas the control group was fed *ad libitum* D12329 diet only. PMI 5011 diet was prepared by making a fine powder of the defined low-fat diet with a Cuisinart food processor. 1% (w/w) of PMI 5011 extract was then blended into the powder of low-fat diet. 100 mLs of water was added and the mixture was turned by hand and flattened with a rolling pen in a large 1 gallon Ziploc bag. The bag was then scored into 1x1 cm squares and placed in the -20ºC freezer. Mice were given squares of PMI 5011 diet from -20ºC storage every other day when food was weighed and replaced to maintain the freshness of the PMI 5011 diet.

2.8 Rodent Tissue Isolation

At the end of the study, both the control and PMI 5011 mice were further divided into 2 groups (N=4) each. Insulin (1.5 U/kg intraperitoneal injection) was given to one half of the mice (control, N=4; PMI 5011, N=4) and an equal volume of physiological saline was administered to the remaining mice (control, N=4; PMI 5011, N=4). The mice were euthanized ninety minutes post-injection and both *vastus lateralis* and *gastrocnemius* skeletal muscle, inguinal and epididymal adipose tissue, heart and liver were collected. All harvested tissues were snap frozen in liquid nitrogen immediately,

and stored at -80°C until isolation of RNA and whole cell extracts and used for further analysis.

2.9 Proteasome Activity Assay

The proteasome activity was assayed using three proteasome substrates as per the manufacturer's instructions (Millipore and Boston Biochemical). The assay kit determines proteasome activity based on recognition of Ac-Leu-Leu-Val-Tyr-AMC (LLVY) for chymotrypsin-like activity, Ac-Arg-Leu-Arg-AMC (RLR) for trypsin-like activity and Ac-Nle-Pro-Nle-Asp-AMC (nLPnLD) for caspase-like activity and is based on detection of the flurophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate. Proteasome activity in C2C12 cells as well as in *gastrocnemius* skeletal muscle was measured using a 20S Proteasome Activity Assay Kit according to the manufacturer's instructions. In brief, the cell and tissue lysates were harvested in 50 mM Tris-Cl, pH 7.4 with 25 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 2 mM ATP, 2 mM PMSF. Caspase-like, chymotrypsin-like and trypsin-like proteasome activity was measured in triplicate by incubating 20 μg per sample of each lysate with a fluorophore 7-Amino-4-methylcoumarin (AMC) labeled peptide substrate nLPnLD-AMC, LLVY-AMC and RLR-AMC and at 37 ^{0}C for 60 min and the free AMC released by proteasome activity was quantified using a 380/460 nm filter set (Molecular Devices, Sunnyvale, CA). Proteasome activity is reported as RFU/μg protein/hr. Each sample was measured in triplicate both in the presence and in the absence of epoxomicin (20 μM), a highly specific 26S proteasome inhibitor (BostonBiochem, Cambridge, MA) to account for any non-proteasomal degradation of the substrate.

2.10 Insulin ELISA Assay

Blood from randomized KK-Ay mice on LFD or PMI 5011 diet (n=8) was collected at baseline, 4 weeks, and 8 weeks and processed as above to collect serum. Insulin levels were determined using an insulin assay kit according to manufacturer's instructions (Downers Grove, IL, USA). Blood insulin levels were measured in duplicate by incubating 2.5 µls of serum sample in an antibody-coated microplate for 2 hours at 4 ºC. Wells were washed and then incubated with anti-insulin enzyme conjugate for 30 minutes at room temperature. Wells were washed and then incubated with enzyme substrate solution for 40 minutes in the dark. The enzyme reaction was stopped with 1 N sulfuric acid and the A_{450} and A_{630} values were measured within 30 minutes of stopping the reaction. Insulin concentrations were calculated using a standard curve based on A_{450} minus A_{630} values.

2.11 Glucose Assay

Blood from the KK-Ay mice on a low fat diet (LFD) or LFD supplemented with PMI 5011 (n=8) was collected at baseline, 4 weeks, and 8 weeks. Glucose levels were determined using a glucose assay kit according to manufacturer's instructions (Ann Arbor, MI, USA). In brief, blood was collected via tail snip, held on ice for 60 minutes for blood to clot and then spun in a centrifuge for 20 minutes at 5,000 x *g*. Blood serum was then separated. Blood glucose was measured in triplicate by incubating 5 µls of serum sample with glucose enzyme mixture at 37ºC for 10 minutes. Absorbance at 500-520 was read to determine glucose levels and calculated as mg/dl based on a standard curve.

2.12 H&E Staining of Skeletal Muscle

At the end of the study, both the control and PMI 5011 mice were further divided into 2 groups (N=4) each. Insulin (1.5 U/kg intraperitoneal injection) was given to one half of the mice (control, N=4; PMI 5011 N=4) and an equal volume of physiological saline was administered to the remainder of the other half of mice (control, N=4; PMI 5011 N=4). The mice were euthanized ninety minutes post-injection and *gastrocnemius* skeletal muscle tissue was harvested and placed in 10% formalin for paraffin embedding and sectioning that was performed by the Pennington Biomedical Research Center Imaging Core. Sections were stained with hematoxylin and eosin stain and slides were scanned using Nanozoom software. Images were then reviewed with the Nanozoom software and the area of cross-sectional segments were analyzed for myofiber size using Image J software.

2.13 Statistical Analysis

GraphPad Prism 5 software was used for statistical analysis of all cell-based and animal- based assays, each completed with a minimum of three replicates. A two-tailed student's *t* test was used to compare means ± SEM for all variables.

2.14 HOMA-IR Analysis

The HOMA-IR or homeostatic model assessment is a method commonly used to quantify and assess insulin sensitivity (Matthews and Hosker 1995). It is a mathematical

equation that relates fasting plasma glucose levels to fasting insulin levels. (fasting Glucose (mmol/L) x fasting Insulin (mU/L) / 22.5). Homeostasis Model of Assessment - Insulin Resistance **(**HOMA-IR) was calculated using the HOMA-IR Calculator version 0.3 based on the formula; fasting Glucose(mmol/L) x fasting Insulin(mU/L) / 22.5 for each individual mouse. http://hcvsociety.org/files/HOMACalc.htm.

CHAPTER 3: *IN VITRO* STUDIES

3.1 Results

A. Dexamethasone-Induced Model of Insulin Resistance *In Vitro*

Glucocorticoids such as dexamethasone decrease the rate of protein synthesis and increase the rate of protein breakdown causing atrophy of skeletal muscle that is under the regulation of insulin and insulin-like growth factor-I (IGF-I) (Goldberg, *et al.*, 1980 and Lofberg *et al.*, 2002). Muscle atrophy is upregulated in insulin resistant states such as those induced by glucocorticoids. The glucocorticoid-induced protein degradation is due to activation of the ubiquitin-proteasome system (Menconi, *et al.,* 2008; Tiao, *et al.,* 1996; Schakman, *et al.,* 2008) and administration of the synthetic glucocorticoid, dexamethasone (DEX), results in increased expression of the two muscle specific E3 ubiquitin ligases Atrogin-1 and MuRF-1, (Menconi, *et al.,* 2008; Sandri, *et al.,* 2004; Stitt, *et al.,* 2004) whose activity leads to muscle atrophy. The Dex-induced increase in Atrogin-1 and MuRF-1 expression is dependent upon ligand binding to the glucocorticoid receptor (Zhao, *et al.,* 2009) leading to the activation of the transcription factors FoxO1and FoxO3a that in turn upregulate the expression of Atrogin-1 (Sandri, *et al.,* 2004; Skurk, *et al.,* 2004) and MuRF-1 (Waddell, *et al.,* 2004). Therefore, the effect of PMI 5011 on components of insulin signaling and the DEX-induced expression of the two E3 ubiquitin ligases, Atrogin-1 and MuRF-1 *in vitro* was examined by treating C2C12 myotubes with the synthetic glucocorticoid dexamethasone at 1 μM for 24 hours. As shown in Figure 3.1, treatment with dexamethasone significantly increased protein expression of both E3 ubiquitin ligases, Atrogin-1 and MuRF-1 compared to controls

(p=0.0008 and p=0.0002 respectively), in agreement with previous studies (Menconi, *et al.,* 2008; Sandri, *et al.,* 2004; Stitt, *et al.,* 2004). These results also demonstrate a significant reduction (p=0.001) in phosphorylation of Akt (serine 473) in the presence of DEX, indicative of insulin resistant states. Sacheck *et al.* (2004), showed that co-incubation of DEX with IGF-1 or insulin *in vitro* blocked DEX-induced myotube atrophy. As demonstrated previously by Ribnicky, *et al.*, 2006, Cefalu, *et al.*, 2008, Wang, *et al.*, 2008 & 2010 and Obanda, *et al.*, 2012, PMI 5011 improves carbohydrate metabolism by enhancing the molecular events of insulin action in skeletal muscle. To test the hypothesis that treatment of the myotubes with increasing concentrations (3 μg/ml-30 μg/ml) of the botanical extract PMI 5011 in the presence of dexamethasone would block the DEX-induced expression of the E3 ubiquitin ligases Atrogin-1 and MuRF-1, C2C12 myotube cells were treated with 1 μM DEX for 24 hours in the presence or absence of PMI 5011 and harvested for whole cell extracts that were subjected to immunoblotting and probed for both Atrogin-1 and MuRF-1 protein expression in addition to phosphorylation of (serine 473) Akt. We found that there was a significant decrease in the DEX-induced expression of Atrogin-1 and MuRF-1 with all three-treatment concentrations of PMI 5011 compared to DEX alone. Importantly, we found that treatment of C2C12 myotubes with 1μM DEX inhibited the phosphorylation of Akt (serine 473) compared to controls cells as seen previously (p=0.001). In the presence of 10 μg/ml PMI 5011 and 30 μg/ml PMI 5011 alone there was a significant increase in phosphorylation of (serine 473) Akt (p=0.0001, p=0.05 respectively) compared to DEX alone without insulin stimulation suggesting that PMI 5011 may act like insulin in that it can activate the phosphorylation of Akt.

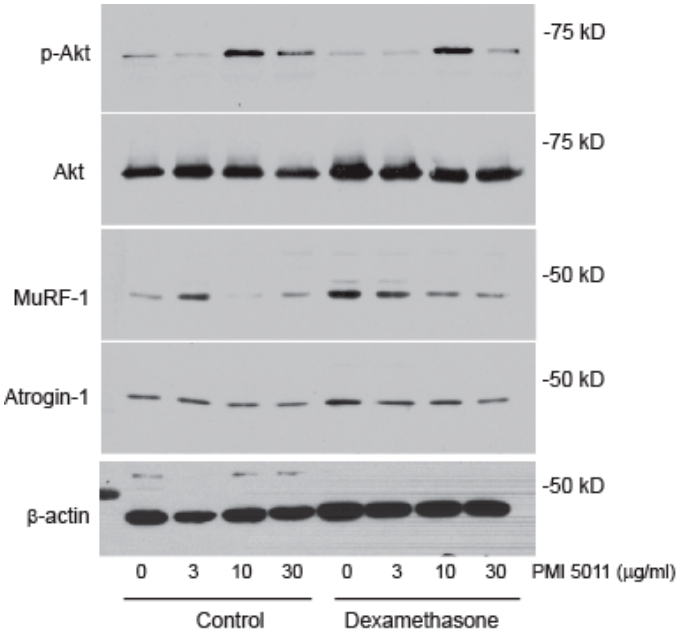


Figure 3.1 Effects of PMI 5011 on p-Akt, MuRF-1 and Atrogin-1 expression in dexamethasone treated C2C12 myotubes. Whole cell extracts were prepared from differentiated C2C12 myotubes treated with and without 1 μM Dex for 24 hours with and without PMI 5011 overnight. 30 μg of the protein extracts were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

We quantified the effect of PMI5011 on the expression of Atrogin-1 and MuRF-1 proteins in the presence of dexamethasone and the effect is reported as a fold change relative to the expression of each ligase under control conditions in Figure 3.2.

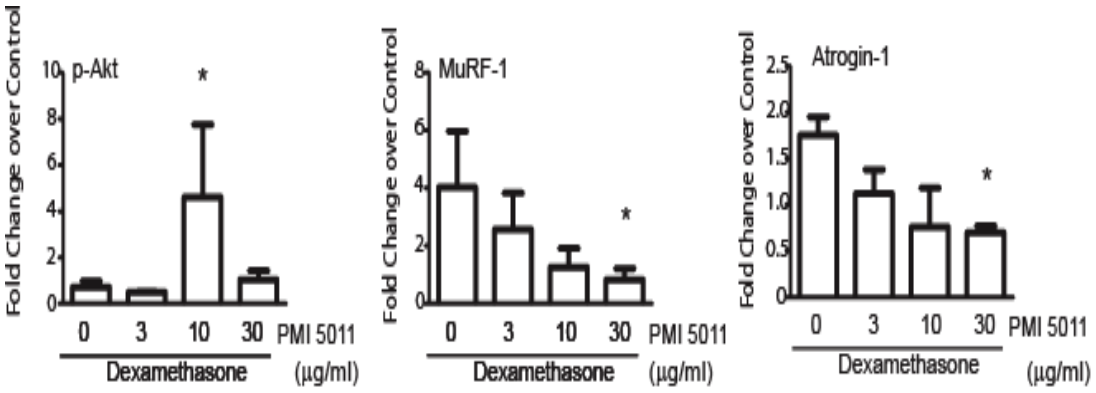


Figure 3.2 Densitometry of western blots on the effects of PMI 5011 on the expression of phospho-Akt, MuRF-1 and Atrogin-1 in Dexamethasone treated C2C12 myotubes. Densitometry was performed using the Un-Scan-It software program. The fold change over control for phospho-Akt, MuRF-1 and Atrogin-1 protein levels were analyzed from three independent experiments, as well as fold changes over DEX with only comparisons for controls shown here for simplicity. The data were reported as the mean -/+ standard deviation. *=P≤0.05. Statistics are un-paired, two-tailed student's *t* test.

To determine the effect of PMI 5011 on *atrogin-1* and *MuRF-1* mRNA levels in this system, myotubes were again treated with 1 μM dexamethasone for 24 hours and treated with or without PMI 5011 overnight and then harvested for RNA isolation. RNA was reverse transcribed and cDNA was used for Quantitative real-time PCR analysis. As shown in Figure 3.3A, in the presence of 1 μM dexamethasone we observed a significant increase ($p=0.0002$) in *atrogin-1* mRNA expression (normalized to cyclophilin B) compared to control cells. Treatment with the botanical extract PMI 5011 at 10 μg/ml significantly ($p=0.003$) decreased this dexamethasone-induced increase in *atrogin-1* mRNA levels. PMI 5011 had no effect on *atrogin-1* mRNA levels alone. As shown in Figure 3.3B, in the presence of PMI 5011 alone, we observed a significant decrease ($p=0.001$) in *MuRF-1* mRNA expression compared to control cells. When treated with 1 μM Dex, *MuRF-1* expression was significantly upregulated ($p=0.0002$) as compared to untreated control cells and again we observed that treatment with 10 μg/ml PMI 5011 significantly ($p=0.0002$) decreased the dexamethasone-induced increase in *MuRF-1* mRNA expression levels. On both the protein and mRNA level, PMI 5011 is able to regulate the DEX-induced expression of these two key mediators of skeletal muscle atrophy as well as enhancing the phosphorylation of Akt the serine-threonine protein kinase that is a key determinant of FoxO-mediated transcriptional regulation of both atrogin-1 and MuRF-1.

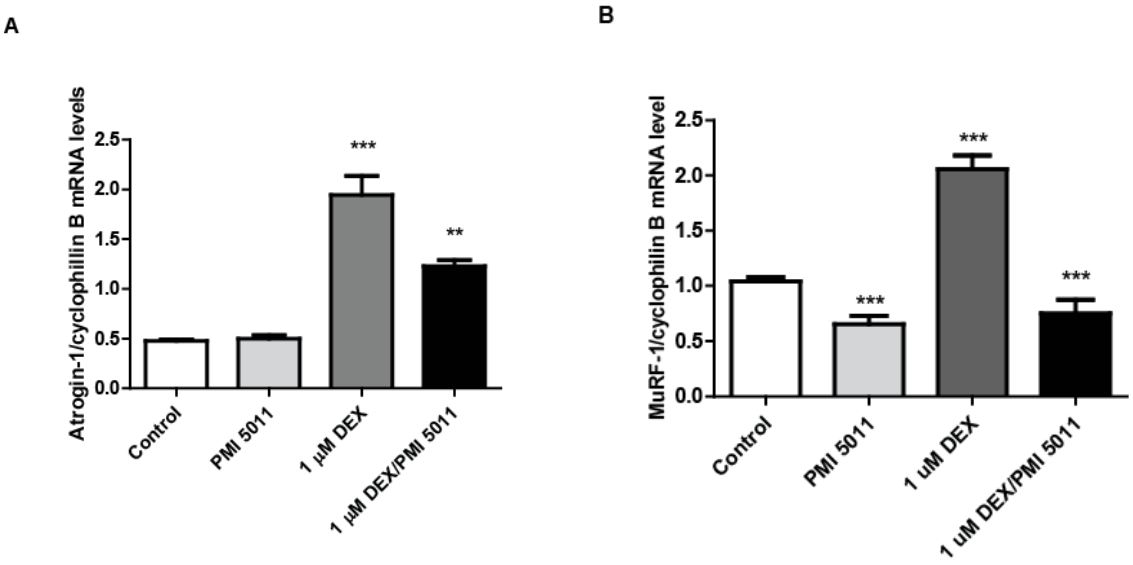


Figure 3.3 The mRNA expression of *atrogin-1* and *MuRF-1* in DEX and PMI 5011 treated myotubes. RNA extracts were prepared from C2C12 myotubes treated with and without 1 μM Dex for 24 hours with and without PMI 5011 at 10 μg/ml overnight. 1 μg of RNA was reverse transcribed and cDNA was analyzed with qRT-PCR for *atrogin-1* and *MuRF-1* mRNA expression normalized to *cyclophillin B*. The data are reported as the mean -/+ standard deviation from three independent experiments. (A) *** = $p<0.001$ at 1 μM Dex compared to control; ** = $p<0.01$ at 1 μM Dex/PMI 5011 compared to 1 μM Dex. (B) PMI 5011 alone *** = $p<0.001$ compared to control; 1 μM Dex *** = $p<0.001$ compared to control; 1 μM Dex /PMI 5011 *** = $p<0.001$ compared to 1 μM Dex alone.

B. Free Fatty Acid-Induced Model of Insulin Resistance *In vitro.*

FFA-induced insulin resistance occurs predominately in skeletal muscle (Boden, 2006). A gradual increase in triglycerides, diacylglycerol (DAG) and the long chain acyl-CoA within muscle cells caused by elevated FFAs is thought to be the underlying cause of insulin resistance in skeletal muscle. This accumulation leads to inhibition of PI3K activation of Akt, thereby inhibiting insulin signaling. Chavez *et al.*, 2005, showed that in C2C12 myotubes, free fatty acids (FFA) inhibited the insulin stimulation of Akt/PKB signaling that regulates glucose uptake in addition to protein synthesis and the transcriptional regulation of both Atrogin-1 and MuRF-1 atrogenes involved in skeletal muscle atrophy. To determine the effect of PMI 5011in another model of insulin

resistance *in vitro*, we treated C2C12 myotubes with palmitic acid. Previous studies showed the saturated FFA palmitate inhibits insulin signaling in C2C12 myotubes as measured by phosphorylation of Akt (Chavez, *et al.,* 2005). Therefore, the C2C12 myotubes were treated with 200μM palmitic acid in the presence or absence of PMI 5011 overnight. The following day, the cells were serum deprived for 2 hours prior to insulin stimulation with 100nM insulin for 4 hours before harvesting for whole cell extracts and RNA. As shown in Figure 3.4 and quantified in Figure 3.5, treatment with 200μM palmitic acid significantly increased both Atrogin-1 and MuRF-1 ($p=0.0008$ and $p=0.0005$ respectively) protein expression as compared to control cells while blocking insulin-stimulated phosphorylation (serine 473) of Akt ($p=0.0006$), confirming that palmitate induced insulin resistance in the C2C12 myotubes in our experiments. In the presence of PMI 5011, the palmitate-induced expression of both Atrogin-1 and MuRF-1 protein levels was significantly lowered ($p=0.004$ and $p=0.05$ respectively) compared to palmitate alone-induced expression of these two atrogenes. In addition, , as seen in dexamethasone-induced insulin resistance, Atrogin-1 and MuRF-1 protein expression levels were significantly lowered in the presence of insulin combined with PMI5011 (10 μg/ml) than with insulin alone ($p=0.001$ and $p=0.008$ respectively). This again demonstrates that PMI 5011 is able to enhance the effects of insulin in this FFA-induced insulin resistant model. In conjunction with decreased levels of palmitate-induced expression of both Atrogin-1 and MuRF-1, treatment with PMI 5011 in the presence of insulin results in a significant increase in phosphorylation (serine 473) of Akt ($p=0.0002$). This is the most exciting result in that insulin alone is unable induce phosphorylation of Akt in palmitate-induced insulin resistance. Additionally, PMI 5011 enhances the action

of insulin, restoring an insulin sensitive state as measured by Akt phosphorylation. The effect of PMI5011 extended to control cells not treated with palmitate. We observed that treatment with 10 µg/ml of PMI 5011 in the presence of insulin increased the levels of p-Akt ($p=0.0002$) over that of insulin stimulation alone suggesting that PMI 5011 is able to enhance the activity of insulin in myotubes in both insulin sensitive and, more important, insulin resistant states.

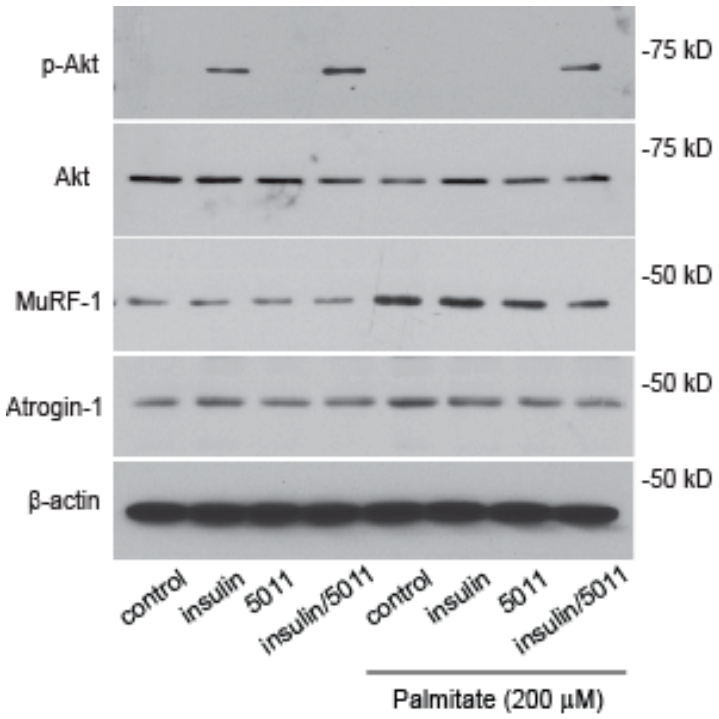


Figure 3.4 The effects of PMI 5011 on phospho-Akt, MuRF-1 and Atrogin-1 expression in free fatty acid induced insulin resistant C2C12 myotubes. Whole cell extracts were prepared from differentiated C2C12 myotubes treated with and without 200 µM palmitic with and without PMI 5011 overnight. Cells were serum deprived for 2 hours prior to stimulation with 100 nM insulin for 4 hours prior to harvest. 30 µg of extracts were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

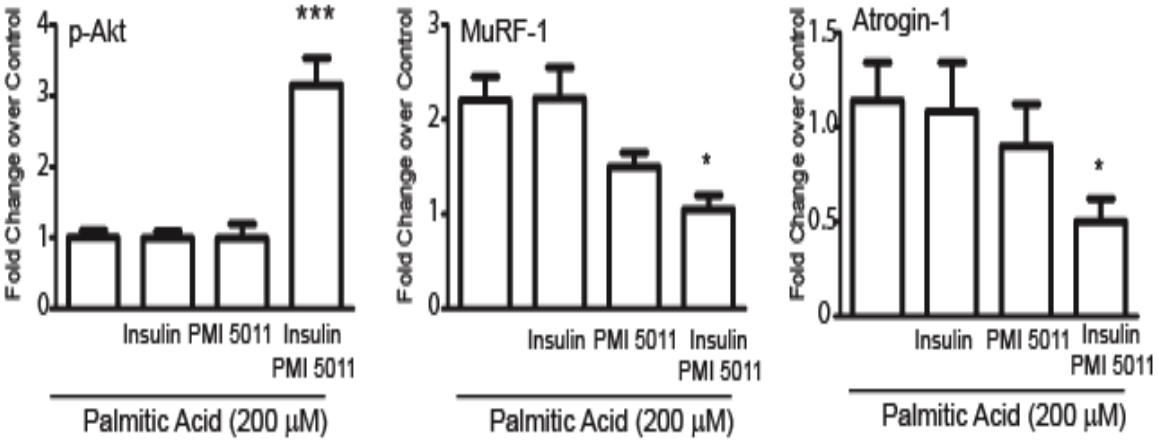


Figure 3.5 Densitometry of the western blots demonstrating the effects of PMI 5011 on p-Akt, MuRF-1 and Atrogin-1 expression in free fatty acid induced insulin resistant C2C12 myotubes. Densitometry was performed using Un-Scan-It software program and statistics were performed using GraphPad Prism software. The fold change over control for phospho-Akt, MuRF-1 and Atrogin-1 protein levels were analyzed from three independent experiments, as well as for fold changes over PA alone. For simplicity sake, only comparisons for fold change over control are shown here. The data were reported as the mean -/+ standard deviation. *=P≤0.05, ***=p≤0.0001. Statistics are un-paired, two-tailed student's *t* test.

Next, to examine the effects of PMI 5011 on mRNA expression levels of *atrogin-1* and *MuRF-1*, myotubes were again treated with 200μM palmitic acid overnight in the presence or absence of PMI 5011. The following day, cells were serum deprived for 2 hours prior to insulin stimulation with 100nM insulin for 4 hours before harvesting for RNA extracts. RNA was isolated, reverse transcribed and cDNA was used for Quantitative real-time PCR analysis. As shown in Figure 3.6A, *atrogin-1* mRNA expression was significantly upregulated (p=0.02) in the presence of palmitate, compared to control cells. In the presence of insulin alone, PMI 5011 alone, and in the presence of both PMI 5011 and insulin FFA-induced *atrogin-1* mRNA expression levels were significantly downregulated (p=0.0002, p=0.0004, and p=0.0001 respectively) compared to palmitate treatment alone. As shown in Figure 3.6B, *MuRF-1* mRNA expression was not affected by the presence of palmitic acid or PMI 5011 compared to controls.

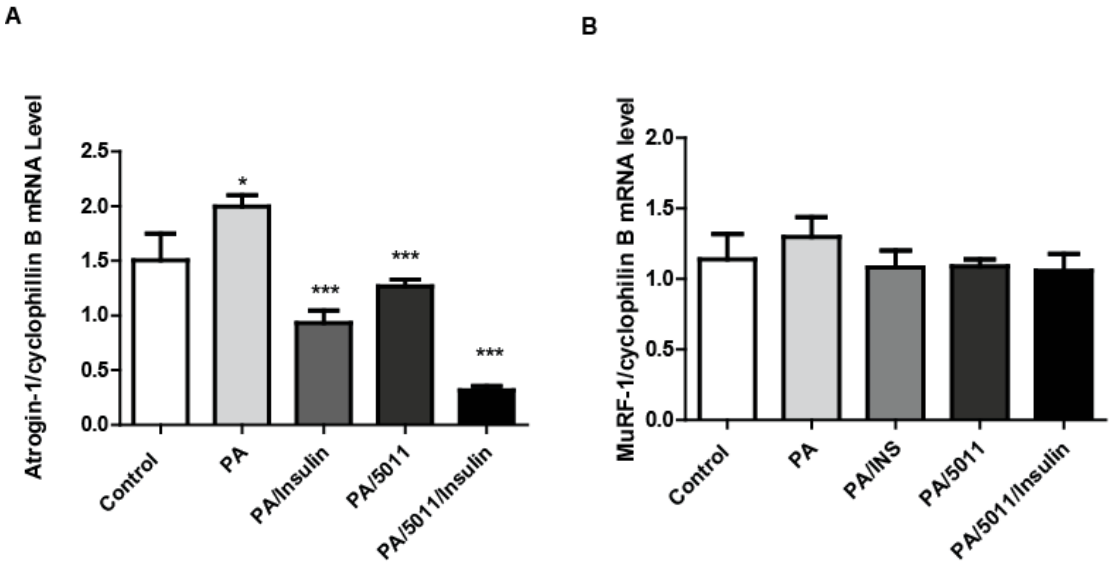


Figure 3.6 The mRNA Expression of *atrogin-1* and *MuRF-1* in Free Fatty Acid-Induced Insulin Resistant Myotubes Treated with PMI 5011. RNA extracts were prepared from C2C12 myotubes treated with and without 200 μM palmitate (PA) with and without PMI 5011 overnight. Cells were serum deprived for 2 hours prior to stimulation with 100 nM insulin (INS) for 4 hours prior to harvest. 1 μg of RNA was reverse transcribed and cDNA was analyzed with qRT-PCR for *atrogin-1* and *MuRF-1* mRNA expression normalized to *cyclophilin B*. The data are reported as the mean -/+ standard deviation from three independent experiments. (A) * = $p<0.02$ at 200 μM PA compared to control; ***= $p<0.0002$ at 200 μM PA/INS compared to 200 μM PA alone; 200 μM PA /PMI 5011 *** = $p<0.0004$ compared to PA alone; PA/5011/INS *** = $p<0.0001$ compared to PA alone.

Next, to answer the question does PMI 5011 alter the effects of insulin on proteasome activity and ubiquitylation *in vitro* these activities were examined. As shown in Figure 3.7A, proteasomal activity is significantly decreased in C2C12 myotubes in the presence of insulin, PMI 5011 and in the presence of both insulin and PMI 5011. This demonstrates that PMI 5011 modulates proteasome activity alone or by enhancing insulin's effects on regulation of proteasomal activity. In all conditions, inhibition of PI3K signaling is associated with increased proteasomal activity. This indicates that PMI 5011 enhances the effects of insulin on proteasomal activity that is PI3K-dependent. In addition, we studied the effects of PMI 5011 on ubiquitylation levels in the myotubes.

We found that the levels of steady-state ubiquitylation were substantially reduced in the presence of both insulin and PMI 5011. However, in the presence of PMI 5011 alone or insulin alone, the levels of ubiquitylation were unchanged as shown in Figure 3.7B. In the presence of the PI3K inhibitor wortmannin, the effects of PMI 5011 on ubiquitylation are blocked, indicating that the observed decrease in ubiquitylation in the presence of PMI 5011 and insulin require the activation of PI3K.

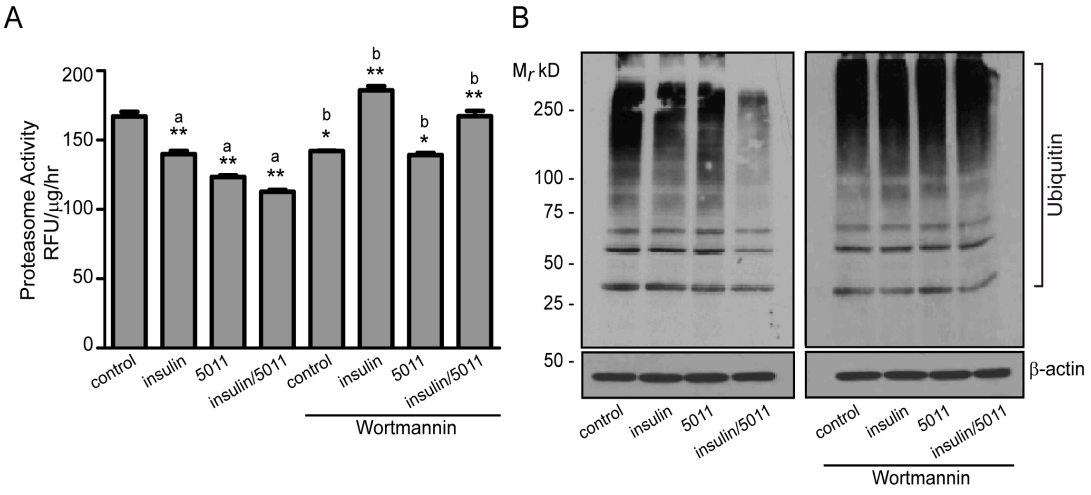


Figure 3.7 PMI 5011 Enhances the Effect of Insulin on Proteasome Activity and Inhibits Ubiquitylation in Myotubes. C2C12 myotubes were treated with 10 µg/ml of PMI 5011 overnight. The cells were then incubated with wortmannin (200 nM) for 1 hour and then treated with insulin (100 nM) for 2 hours. (A) The cells were harvested and assayed for the proteasomal chymotrypsin-like protease activity. MgATP was added to the lysis buffer to maintain the 26S structure of the proteasome. The data is reported as the mean -/+ standard deviation from three independent experiments. A=compared to control; b=compared to related treatment (-) wortmannin; * = $p<0.05$, ** = $p<0.01$. (B) Whole cell extracts were subjected to SDS-PAGE followed by western blot analysis using an anti-ubiquitin antibody to assay the level of generalized ubiquitylation. The data are representative of three independent experiments.

3.2 Discussion

In light of the previous findings demonstrating that PMI 5011 enhances insulin signaling and increases protein in skeletal muscle from KK-A^y mice, a murine model of insulin resistance (Wang, *et al.,* 2011), we hypothesized that the muscle atrophy associated with type 2 diabetes could be attenuated with treatment of PMI 5011. We predicted that PMI 5011 would do so by decreasing protein degradation, either through regulation of the two-muscle specific E3 ubiquitin ligases Atrogin-1 and MuRF-1 or by directly regulating proteasome activity. Because various models of skeletal muscle atrophy show increases in E3 ubiquitin ligases *atrogin-1* and *MuRF-1* gene expression (Lecker, *et al.,* 2004) we chose to evaluate the effect of PMI 5011 on the expression of these two ligases in two highly accepted models of insulin resistance associated with muscle atrophy: glucocorticoid and FFA-induced models of insulin resistance. Our *in vitro* studies using C2C12 myotubes show that PMI 5011 indeed has significant effects on the expression of these two ligases in insulin resistance. Studies have shown that administration of the synthetic glucocorticoid, dexamethasone (DEX), results in an increased expression of the two E3 ubiquitin ligases; Atrogin-1 and MuRF-1 (Menconi, *et al.,* 2008; Sandri, *et al.,* 2004; Stitt, *et al.,* 2004) that leads to myotube atrophy. Indeed, we found that treatment of myotubes with DEX increased levels of both ligases. On the protein and mRNA level, PMI 5011 decreased the glucocorticoid-induced expression of MuRF-1. The proteasome is unable to degrade large myofibrillar proteins that are the primary group of targeted proteins in skeletal muscle associated with atrophy (Munoz, *et al.,* 1993). MuRF-1 dependent ubiquitylation of skeletal muscle proteins accounts for the large majority of ubiquitin modifications associated with muscle atrophy and it has been

shown to interact directly with and regulate the ubiquitylation of several myofibrillar proteins (Cohen, *et al.,* 2009; Clarke, *et al.,* 2007). Our data is consistent with PMI 5011 regulation of MuRF-1 in our hormone-induced model of insulin resistance and muscle atrophy. Our second model of insulin resistance mimics the elevated physiological level of free fatty acids that causes muscle (peripheral), hepatic and vascular insulin resistance (Boden, 1997). We found that treatment with palmitic acid significantly increased the levels of both Atrogin-1 and MuRF-1 protein expression and completely blocked the phosphorylation of Akt confirming our model of insulin resistance in C2C12 myotubes. In the presence of PMI 5011 and insulin, phosphorylation of Akt was restored although insulin alone and PMI 5011 alone were unable to induce phosphorylation of Akt in palmitate-induced insulin resistant myotubes. This indicates that PMI 5011 is able to restore insulin sensitivity to the cells. Unlike the DEX-induced model of insulin resistance reported here, *MuRF-1* gene expression was unaffected by PMI 5011 treatment in palmitate-induced insulin resistant myotubes. In fact, there was little effect of palmitic acid on induction of *MuRF-1* gene expression in this model suggesting a lesser role of MuRF-1 in FFA-induced insulin resistance. Perhaps this is due to the more subtle skeletal muscle atrophy associated with insulin resistance of type 2 diabetes than what is observed in glucocorticoid-induced muscle atrophy models since MuRF-1 is the main ligase involved in degradation of larger myofibrillar proteins. *Atrogin-1* gene expression was however, significantly upregulated with palmitate and in the presence of both PMI 5011 and insulin this FFA-induced gene expression was attenuated. Atrogin-1 may play a larger role in the sometimes more subtle atrophy associated with type 2 diabetes.

Overall, PMI 5011 enhances phosphorylation of Akt the potent serine-threonine kinase that is a dynamic regulator of insulin signaling, skeletal muscle hypertrophy and transcriptional regulator of atrogene expression. The mechanisms by which phospho-Akt targets Atrogin-1 or MuRF-1 in this system is yet to be determined. We know that phosphorylation of Akt leads to the inhibition of FoxO transcription factors that in turn are excluded from the nucleus where they can no longer upregulate the expression of Atrogin-1 and MuRF-1. However, the effects of PMI 5011 on the expression levels of these transcription factors have not been evaluated to date *in vitro*. In addition, it is also known that insulin also activates the mTOR pathway, leading to an increase in protein synthesis that may regulate the rate of protein degradation through "cross-talk" with the ubiquitin-proteasome system (Lagirand-Cantaloube, *et al.,* 2008). This may extend to the down regulation of Atrogin-1 and MuRF-1 that is observed with treatment of PMI 5011 in the presence of insulin. Another possible mechanism by which PMI 5011 regulates protein degradation is via the translation initiation factor eIF3f that is a known substrate and target of Atrogin-1 (Lagirand-Cantaloube, *et al.,* 2008). PMI 5011 decreases both protein and mRNA levels of Atrogin-1, and it is therefore possible that PMI 5011 also increases protein content by indirectly blocking the degradation of eIF3f by downregulating Atrogin-1 expression. We found that PMI 5011 enhanced insulin signaling in a way that may help to maintain skeletal muscle mass via restoration of the effect of insulin on protein degradation in skeletal muscle. These *in vitro* studies in C2C12 myotubes suggest a potential role for PMI 5011 in regulating muscle specific ubiquitin ligases involved with the ubiquitin proteasome and protein degradation in skeletal muscle. PMI 5011 may have therapeutic implications for the treatment of

muscle loss in a range of catabolic diseases including insulin resistance associated with type 2 diabetes. The following chapter will discuss the effects of PMI 5011 in regulating skeletal muscle atrophy in a murine model of insulin resistance *in vivo.*

CHAPTER 4: *IN VIVO* STUDIES

4.1 Results

To examine the *in vivo* effects of PMI 5011 on the activity of the ubiquitin proteasome system, a murine model of obesity-related insulin resistance and diabetes known as KK.Cg-A^y/+ mice was used. The inbred mouse strain KK, established in Japan as a diabetic strain, develops non-insulin-dependent diabetes mellitus with mild obesity, mainly due to insensitivity of the peripheral tissue to insulin (Suto, *et al.*, 1998). Diabetes and obesity in the KK mouse alone is fairly moderate but introducing the A^y allele (KK-A^y) worsens the pathophysiological condition of overt diabetes with accompanying hyperinsulinemia. In the KK-A^y mouse strain, diet-induced obesity is caused by a mutation of the yellow obese gene A^y that leads to insulin resistance of the adipose tissue. This leads to glucose intolerance, insulin resistance and the development of diabetes in the mice that is secondary to diet-induced obesity or aging (Ikeda, 1994). The mechanism for obesity caused by the A^y allele is thought to be due to the agouti peptide. Ectopic expression of the agouti peptide can act as an antagonist to the melanocortin 4 receptor and inhibit the action of α-melanocyte-stimulating hormone signals (Huszar, *et al.*, 1997). It is not clear whether the diabetic and hyperglycemic effects related to the Ay allele occur in a simple additive manner or whether impairments result from complex interactions between diabetic genes already present in the genome (Suto, *et al.*, 1998). Obesity-related insulin resistance that precedes hyperglycemia, hyperinsulinemia, glucose intolerance and dyslipidemia and the resulting metabolic complications (Mittenforfer, 2011) occurs in numerous tissues including skeletal muscle

tissue. The KK-A^y model therefore provides a model for observing the effects on PMI 5011 on skeletal muscle protein degradation associated with obesity-related insulin resistance. This mouse model of insulin resistance and diabetes was used in earlier studies that established PMI 5011 regulates glucose metabolism and insulin signaling in skeletal muscle (Wang, *et al.,* 2011). Therefore, we carried out our studies on the effect of PMI 5011 on the ubiquitin-proteasome system in skeletal muscle using the KK-A^y strain. Figure 4.1., shows the study design. At the end of the study at week 12 tissues were harvested for gene expression analysis, protein analysis, proteasomal and non-proteasomal activity.

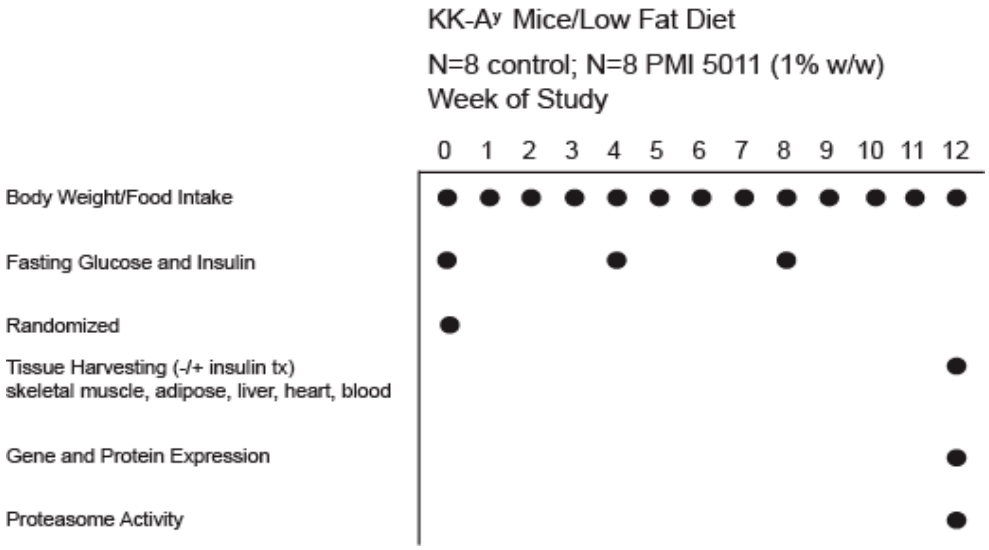


Figure 4.1 *In vivo* Study Design. Male KK-A^y mice (n=18) were singly housed and randomly divided into a control group (N=9) and a PMI 5011-treated group (5011; n=9). Body weight and food intake measurements were taken weekly. Baseline, week 4 and 8 were fasting glucose and blood serum collection for insulin ELISA. Study ended at week 12.

As part of our data collection, body weight measurements were taken weekly on each individual mouse. As shown in Figure 4.2., by week 8 those animals on PMI 5011 had a small, but significant increase in body weight compared to the control animals. In previous studies by Wang *et al.,* 2011 there was no effect on body weight in KK-A^y mice on the PMI 5011 diet compared to control animals.

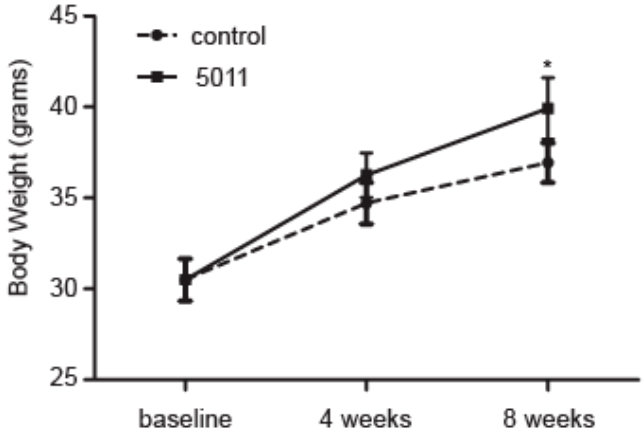


Figure 4.2 Body Weight Measurements. Body weights of individual mice were taken weekly. Mice were measured in grams. PMI 5011 mice were slightly, but significantly heavier than control mice on LFD ($p \leq 0.05$).

Food intake measurements were also measured weekly on individual mice. As shown in Figure 4.3, the food intake for the PMI 5011supplemented animals was slightly ($p<0.05$) higher than control mice. This may account for the slight increase in body weight of the PMI 5011 mice.

Although there was a slightly higher body weight in the PMI 5011 fed mice, this weight gain did not correlate with higher blood glucose (Figure 4.4) or insulin levels (Figure 4.5) in these animals. Serum samples were analyzed for glucose levels with a colorimetric hexokinase glucose assay according to manufacturer's instructions. As shown in Figure 4.4, by week eight the PMI 5011 supplemented animals had a

significantly lower blood glucose level compared to the control mice, in agreement with previous studies (Wang, *et al.,* 2011).

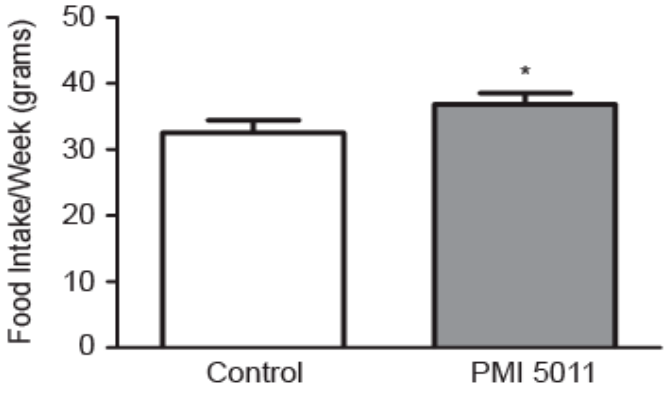


Figure 4.3 Food Intake Measurements. Food intake of individual mice was measured weekly. Weights were measured in grams. PMI 5011 mice ate significantly ($p<0.05$) more food than control mice on the low fat diet (Control).

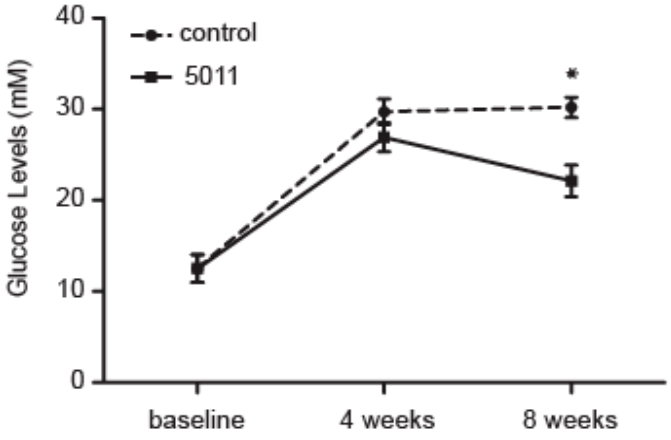


Figure 4.4 Fasting Blood Glucose Levels. At baseline, 4 weeks, and 8 weeks, individual mice were fasted for a period of 4 hours. Blood serum samples were taken via tail tip bleed for blood glucose analysis using a glucometer. PMI 5011 treated mice had significantly lower ($p\leq0.05$) fasting glucose levels as compared to control LFD mice by week 8.

Blood insulin levels were assayed by insulin ELISA for relative insulin levels according to manufacturer's instructions. As shown in Figure 4.5, in accordance with

previous studies (Wang, *et al.,* 2011) , we found that those mice on the PMI 5011 diet had significantly lower levels of insulin by week 8 as compared to control mice on a low fat diet alone.

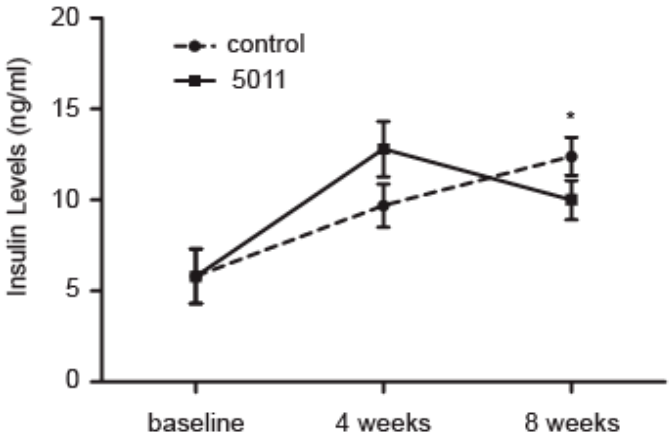


Figure 4.5 Fasting Serum Insulin Levels. At baseline, 4 weeks, and 8 weeks, individual mice were fasted for a period of 4 hours. Blood serum samples were taken via tail tip bleed for blood insulin analysis in an insulin ELISA assay. PMI 5011 treated mice had significantly lower ($p \leq 0.05$) fasting insulin levels as compared to control LFD mice by week 8.

To assess the effect of PMI 5011 on insulin sensitivity in this highly insulin resistant model, blood serum samples were obtained after a four-hour fast via tail tip bleeds. In Figure 4.6, and in accordance with previous studies by Wang *et al.*, 2011, we found that the PMI 5011 supplemented mice had significantly lower HOMA-IR levels by week 8 as compared to control mice on LFD alone. This reconfirmed that a defined low-fat diet containing 1% (w/w) of PMI 5011 increased glucose disposal in a murine model that is glucose intolerant, insulin resistant, and has overt diabetes as seen previously (Wang *et al.,* 2011).

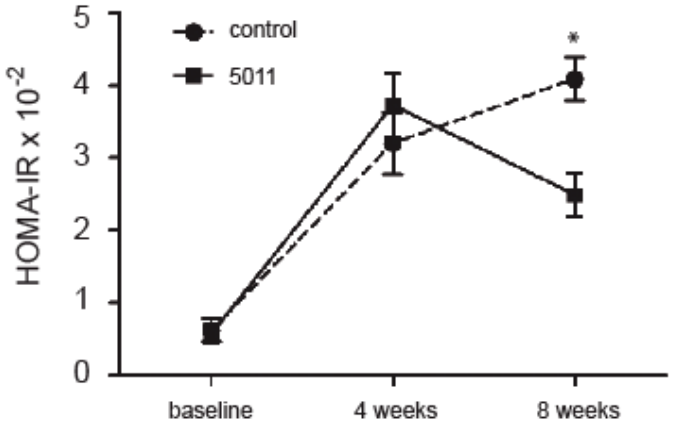


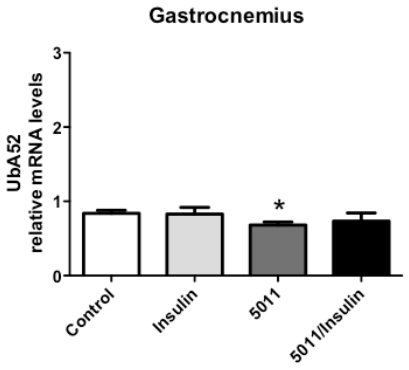
Figure 4.6 Fasting **HOMA-IR Levels.** At baseline, 4 weeks, and 8 weeks, individual mice were fasted for a period of 4 hours. Blood serum samples were taken via tail tip bleed for blood insulin analysis in an insulin ELISA assay, as well as, blood glucose by glucose assay. HOMA-IR was calculated using a standard formula of Glucose X Insulin/22.5 = Glucose in Molar units (mmol/L). PMI 5011 treated mice had significantly lower ($p \leq 0.05$) fasting HOMA-IR levels as compared to control LFD mice by week 8.

Insulin resistance associated with type 2 diabetes has been linked to skeletal muscle loss, a condition known as muscle atrophy (Park, *et al.,* 2009). Sishi, *et al.,* 2010 demonstrated that diet-induced obesity leads to skeletal muscle atrophy and previous studies in the KK-A^y murine model of obesity and diabetes showed that a diet supplemented with the botanical extract PMI 5011 leads to an increase in skeletal muscle protein content (Wang, 2011). Additionally, Lecker *et al.,* 2004, found that multiple types of skeletal muscle atrophy involve a common program of gene expression changes. In particular, they found that polyubiquitins, Ub fusion proteins, ubiquitin ligases Atrogin-1 and MuRF-1, several subunits of the 20S proteasome in addition to cathepsin L were commonly activated. Specifically, in STZ-induced diabetic rats they found that the levels of UbA, UbB, UbC, the ubiquitin-conjugating enzyme E2 variant 1 (Ube2v1), ubiquitin ligases Atrogin-1 and MuRF-1and proteasomal subunits PSMA5 and PSMB3 were all elevated.

In insulin resistant states, there is impaired signaling of the PI3K/Akt signaling pathway that leads to an increase in ubiquitin-proteasome-dependent protein degradation (Wang, *et al.,* 2006). In the first committed step of this system, ubiquitin, an abundant 8 kDa protein highly conserved in all eukaryotes and responsible for the post-translational modification to other proteins is covalently attached to its substrate by glycine on its C-terminal residue to the lysine side chain of target proteins that results in an isopeptide bond (Kercher, *et al.,* 2006). Through several lysine residues onto ubiquitin itself, additional ubiquitins moieties are added in a polyubiquitin chain that is the classic target signal for degradation in the 26S proteasome (Wing, *et al.,* 2011). This conjugation of ubiquitin to target proteins involves ATP-dependent sequence of reactions that are catalyzed by three enzymes; first, the E1 ubiquitin activating enzyme, second, the E2 ubiquitin conjugating enzyme, and third, the E3 ubiquitin ligases that provides substrate specificity to the reaction (Pickart, 2004). Hershko *et al.,* 1982, first discovered this complex system in rabbit reticulocyte extracts in 1982. To date, there are approximately 30 genes encoding E2 ubiquitin conjugating enzymes and nearly 900 genes encoding E3 ubiquitin ligases. There are four known ubiquitin genes. Two of these genes encode polyubiquitin and two encode ubiquitin-ribosomal fusion proteins (Wing, *et al.,* 2011). Lecker, *et al.*(2004), found that in STZ-induced diabetic rat that UbA, UbB, and UbC were upregulated by at least two fold in this model of diabetes. Based on this information, we selected ubiquitin B, ubiquitin C, and Ubiquitin A-52 ribosomal fusion protein that showed a 2.7, 2.9, and 2.0 fold change increase respectively in *gastrocnemius* skeletal muscle from rats with diabetes (Lecker, *et al.*, 2004) to assay the effects of PMI 5011 supplementation. We found that mice supplemented with PMI 5011 had a slight,

although significant decrease in UbA52 gene expression (*=p≤0.05) compared to control mice but had no effect on Ubiquitin B or Ubiquitin C expression as shown in Figure 4.7(A-C).

A.



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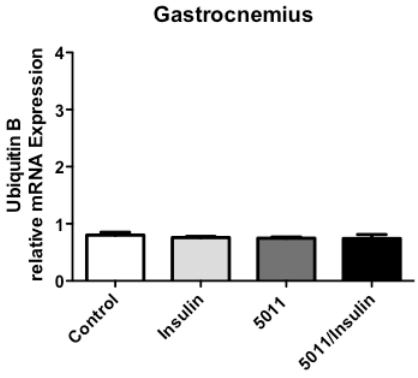

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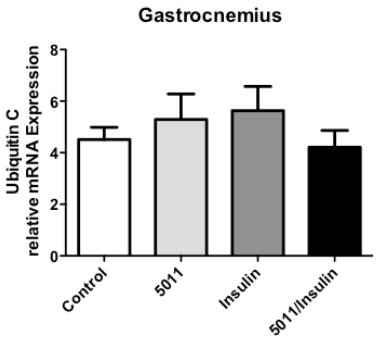


Figure 4.7 PMI 5011 Regulates UbA52 Gene Expression, but Not Ubiquitin B or C. RNA was isolated from skeletal muscle from control and PMI 5011 supplemented mice. 1 µg of RNA was reverse transcribed and cDNA was analyzed with qRT-PCR for Gene expression for *UBA52* (A), *UBB* (B), and *UBC* (C) mRNA expression normalized to *cyclophilin B*. Results are reported as the mean -/+ standard deviation (N=4/group). Significance is reported relative to control or insulin treated mice, as indicated. *=p ≤0.05.

Next, we chose to evaluate the effects of PMI 5011 on mRNA expression of Ube2v1 the ubiquitin-conjugating enzyme E2 variant 1 that showed a 3.0 fold change increase in gene expression in *gastrocnemius* skeletal muscle from rats with diabetes (Lecker, *et al.,* 2004). The Ubiquitin E2 Variant (UEV) proteins share sequence and structure similarity to E2 enzymes, however they lack the activity of the E2 enzymes. Sancho, *et al.,* 1998 were unable to demonstrate recombinant Human UEV-1-mediated inhibition of E2 enzyme-catalyzed ubiquitylation, and suggested that UEV proteins function to regulate ubiquitylation of proteins by directly modulating the transfer of ubiquitin to specific substrates through E2 enzymes. It is thought that interactions of E2 enzymes with UEV proteins provide a higher degree of combinatorial possibilities and direct a given enzyme to specific substrates and modulate its activity (Sancho, *et al.,* 1998). As shown in Figure 4.8, PMI 5011 significantly decreased (*=p≤0.05) the mRNA levels of *Ube2v1* in the presence and absence of insulin stimulation compared to control mice

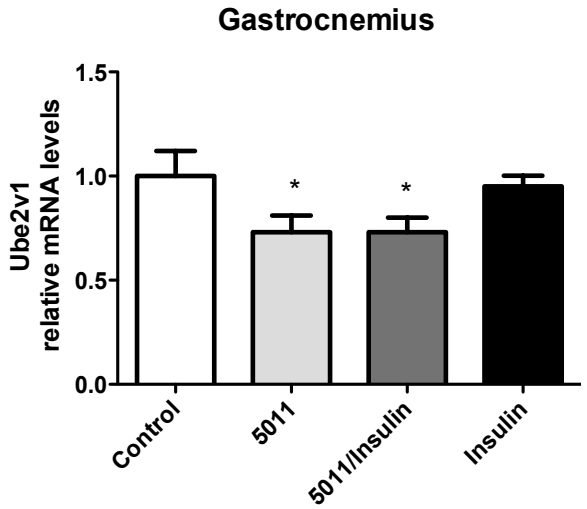


Figure 4.8 PMI 5011 Regulates Gene Expression of Ube2v1, the ubiquitin-conjugating enzyme E2 variant 1. RNA was isolated from skeletal muscle from control and PMI 5011 supplemented mice. 1 µg of RNA was reverse transcribed and cDNA was analyzed with qRT-PCR for Gene expression for *Ube2v1* mRNA expression normalized to *cyclophillin B*. Results are reported as the mean -/+ standard deviation (N=4/group). Significance is reported relative to control or insulin treated mice, as indicated. *=p ≤0.05.

There are two major classes of E3 ubiquitin ligases. One class is the HECT domain class that contains around 90 genes and is known for accepting ubiquitin from the E2 enzymes and transferring the bound ubiquitin to the substrate protein. The other class is the largest class containing nearly 800 genes with conserved RING finger motif that functions by binding both the substrate and E2 enzyme to coordinate the transfer of ubiquitin from the E2 to the targeted substrate. (Pickart, 2004) The RING-type ligases can be further divided into two classes. One group of the RING-type ligases are single subunit proteins and the second group is composed of multi-subunit complexes typified by the family of Skp-Cullin-F-box protein (SCF) ligases that have distinct subunits for substrate recognition and E2 binding functions (Wing, *et al.,* 2011). The two muscle-specific E3 ubiquitin ligases associated with atrophy are Atrogin-1/MAFbx and MuRF-1. Atrogin-1/MAFbx is an F-box containing protein that is part of a SCF ligase complex where the F-box containing subunit is the critical substrate recognition component of the complex (Bodine, *et al.,* 2001 and Gomes, *et al.,* 2001). MuRF-1 is a monomeric ligase with a tripartite RING finger-B box-coiled-coiled motif. Numerous studies have shown that an increase in these two muscle-specific ligases is involved in catabolic conditions associated with muscle atrophy. MuRF-1 has been shown to interact with eight myofibrillar proteins and thick filament proteins (Witt, *et al.,* 2005). In addition, MuRF-1 knock-out mice have normal muscle structure and function while deletion of MuRF-1/MuRF-2 is lethal. After muscle denervation, MuRF-1 -/- mice are significantly less susceptible to muscle loss compared to MuRF-1 wild-type mice (Bodine, *et al.,* 2001). Atrogin-1/MAFbx-1 has also been found to decrease transcription and translation of muscle myofibrillar proteins and it was originally cloned because its mRNA was found to

be the most highly induced in skeletal muscle in catabolic states such as fasting (Gomes, *et al.,* 2001). More important, mice lacking the *atrogin-1* gene also show reduced rates of muscle atrophy (Bodine, *et al.,* 2001). Lecker *et al.*, 2004, found that in studies of rats with diabetes, there is a 9.1 fold increase in *atrogin-1 mRNA* expression levels. Due to these important findings, we studied the effects of PMI 5011 on gene and protein expression in the KK-A^y model of diabetes and insulin resistance. As shown in Figure 4.9A, those KK-A^y mice on the PMI 5011 diet had significantly lower levels ($p \leq 0.05$) of *atrogin-1* expression compared to control mice in the gastrocnemius skeletal muscle. In those animals that received insulin prior to the end of study, PMI 5011 animals also had lower levels of *atrogin-1* mRNA levels compared to control insulin-stimulated mice, although the reduction was not significant. We next assessed the mRNA levels of *MuRF-1* in the KK-A^y mice on the PMI 5011 diet. We found that the PMI 5011 diet significantly reduced ($p \leq 0.05$) levels of *MuRF-1* gene expression compared to control animals as shown in Figure 4.9B. In those animals that were insulin-stimulated prior to tissue harvest, there was no observed effect of PMI 5011 on *MuRF-1* gene expression. Next, to assess the effects of diets enriched with PMI 5011 on the protein expression of Atrogin-1 and MuRF-1 in skeletal muscle, we conducted immunoblotting analyses of skeletal muscle collected from control mice and PMI 5011 supplemented animals, in the absence or presence of insulin treatment. In addition to analysis of Atrogin-1 and MuRF-1, we also assayed the effects of PMI 5011 on insulin signaling by studying the effects on phosphorylation of Akt in the same mice.

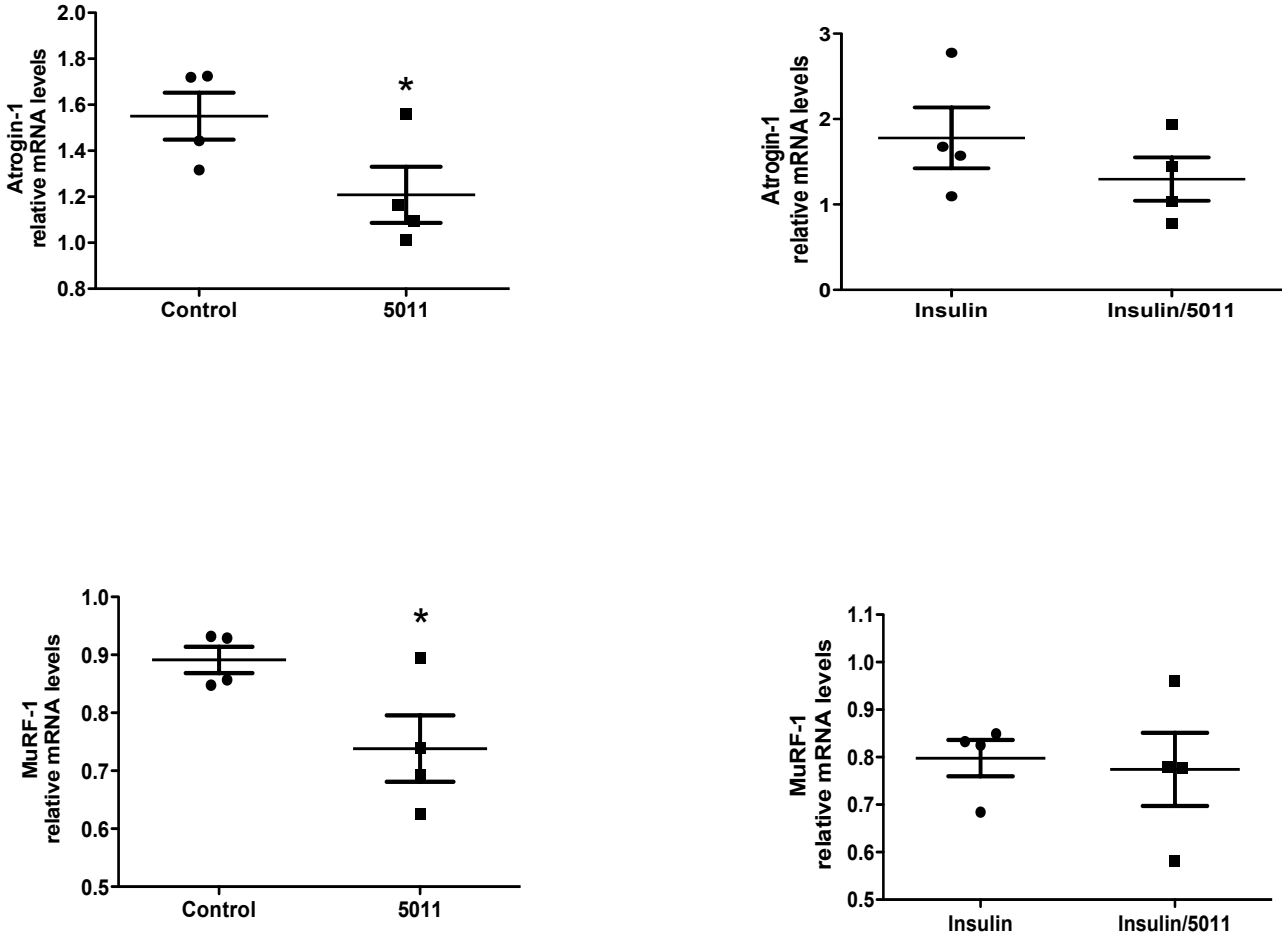


Figure 4.9 PMI 5011 Regulates Atrogin-1 and MuRF-1 Gene Expression in Skeletal Muscle. RNA was isolated from skeletal muscle from control and PMI 5011 supplemented mice. 1 µg of RNA was reverse transcribed and cDNA was analyzed with qRT-PCR for *atrogin-1* (A) and *MuRF-1* (B) mRNA expression normalized to *cyclophilin B*. Results are reported as the mean -/+ standard deviation (N=4/group). Significance is reported relative to control or insulin treated mice, as indicated. *=p ≤0.05.

As shown in Figure 4.10A and 4.10B, PMI 5011 significantly decreased both Atrogin-1($p \leq 0.05$) and MuRF-1($p \leq 0.05$) protein expression in skeletal muscle. In addition, PMI 5011 treatment improved insulin signaling in skeletal muscle as shown by increased phosphorylation of Akt. Importantly, this increase in Akt phosphorylation is associated with decreased expression of both Atrogin-1 and MuRF-1 protein expression. Lastly, activation of Akt correlates with increased phosphorylation of FoxO3a (Figure 4.10A). Akt directly phosphorylates FoxO proteins, leading to inhibition of FoxO transcriptional activity and blocking FoxO-dependent upregulation of both Atrogin-1 and MuRF-1 ubiquitin ligases (Glass, 2005). FoxO1 and FoxO3a are both members of the FoxO class of forkhead transcription factors and are downstream targets of Akt in skeletal muscle. FoxO transcription factors are excluded from the nucleus when they are phosphorylated by Akt. Therefore, phosphorylation of the FoxO proteins leads to decreased gene expression of *atrogin-1* and *MuRF-1* (Sandri, *et al.,* 2004). We sought to determine if the downregulation of both Atrogin-1 and MuRF-1 in addition to upregulation of phospho-Akt protein expression was related to the regulation of FoxO phosphorylation. In order to do this, we measured the levels of total FoxO3a protein in addition to the phosphorylated (serine 253) form of FoxO3a in the control and PMI5011 supplemented, with and without insulin stimulation. We found that PMI 5011 supplementation did correspond with increased phosphorylation of FoxO3a, as shown in Figure 4.10A and 4.10B. When FoxO3a is phosphorylated downstream of p-Akt, it is excluded from the nucleus where it can no longer upregulate the expression of Atrogin-1 or MuRF-1.

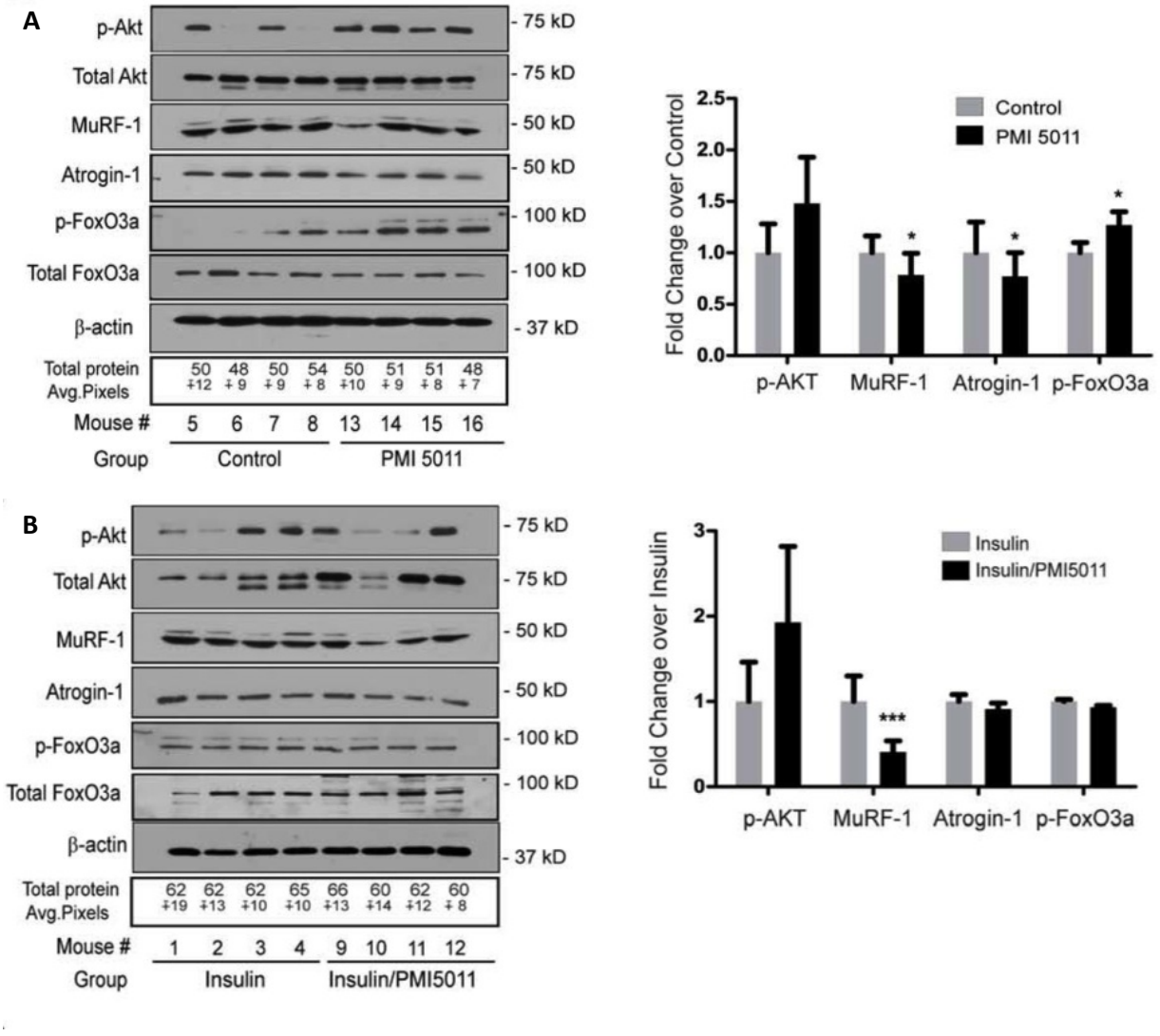


Figure 4.10. PMI 5011 Regulates Atrogin-1, MuRF-1, p-Akt and p-FoxO3a protein expression in skeletal muscle. (A,B) Whole cell extracts were processed from gastrocnemius skeletal muscle from KK-A^y supplemented with and without the botanical extract PMI 5011. Whole cells extracts were analyzed using SDS-PAGE followed by western blot analysis for phosphor-Akt, total Akt, MuRF-1, Atrogin-1, p-FoxO3a, total FoxO3a and β-actin loading controls. Fold change for phopsho-Akt/total Akt, phosphor-FoxO3a/total FoxO3a, MuRF-1/total protein and Atrogin-1/total protein is reported in (A) the PMI 5011 mice relative to control mice and (B) PMI 5011 mice relative to control mice receiving insulin prior to tissue harvest. *=$p \leq 0.05$ and ***= $p \leq 0.001$. Densitometry was performed using the Un-Scan-It software program.

Although we do not provide direct evidence that FoxO3a is excluded from the nucleus in this study, our data is consistent with decreased FoxO3a activity due to phosphorylation, a possibility that is supported by the decrease we observe in gene expression of *atrogin-1* and *MuRF-1*. Future studies to determine cellular localization with both immunoblotting of cytosolic and nuclear fractioned samples in addition to immunolocalization would provide further evidence of PMI5011 regulation of *atrogin-1* and *MuRF-1* expression by AKT-mediated inhibition of FoxO3a activity.

Increased levels of ubiquitylated proteins in atrophying skeletal muscle occurs in many catabolic states, including diabetes (Wing, 2004). The ubiquitin-modified proteins are targeted for degradation at the proteasome and Atrogin-1 and MuRF-1 are the primary ubiquitin ligases responsible for tagging the proteins for degradation. The decrease in Atrogin-1 and MuRF-1 expression would be consistent with reduced levels of ubiquitin modified proteins in the PMI5011 supplemented animals. Therefore, we assessed the general levels of ubiquitylation in control and PMI 5011 supplemented mice. As shown in Figure 4.11, the general ubiquitylation of skeletal muscle proteins are lower in animals supplemented with PMI 5011 (N=4) compared to control animals (N=4). In addition, the effect of insulin stimulation on ubiquitylation is enhanced by supplementation of PMI 5011 (N=4) compared to insulin alone (N=4) (Figure 4.11B**).**

We next assayed the effect of PMI 5011 on the mRNA levels of two specific 20S proteasomal subunits, the proteasome 20S subunit alpha 5 (PSMA5) and 20S subunit beta 3 (PSMB3) that are strongly upregulated with muscle loss (Lecker, *et al.,* 2004) in a study that used microarray analysis to examine the transcriptional adaptations of mRNA

in normal muscle versus atrophying muscle in fasted mice or rats with renal failure, cancer or diabetes (Lecker, *et al.,* 2004).

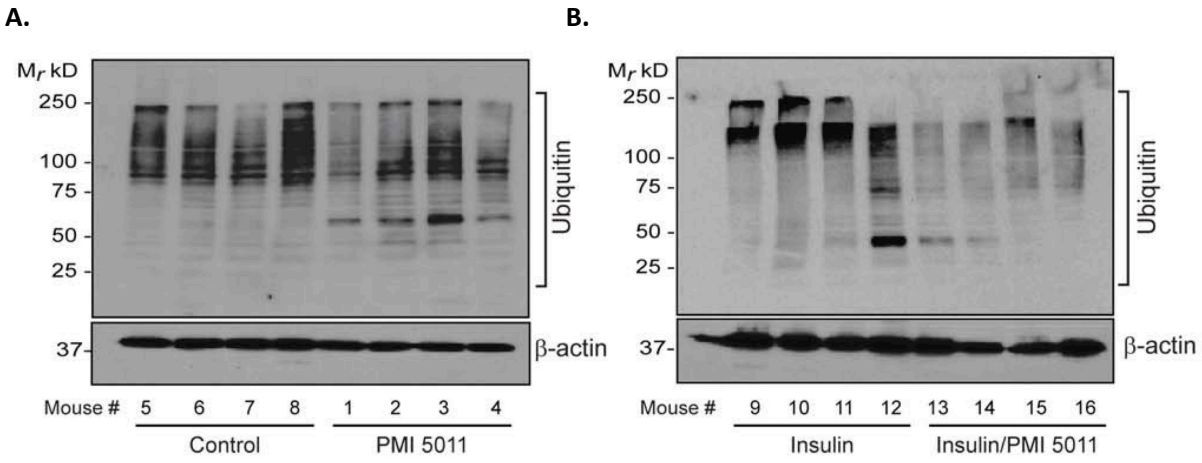
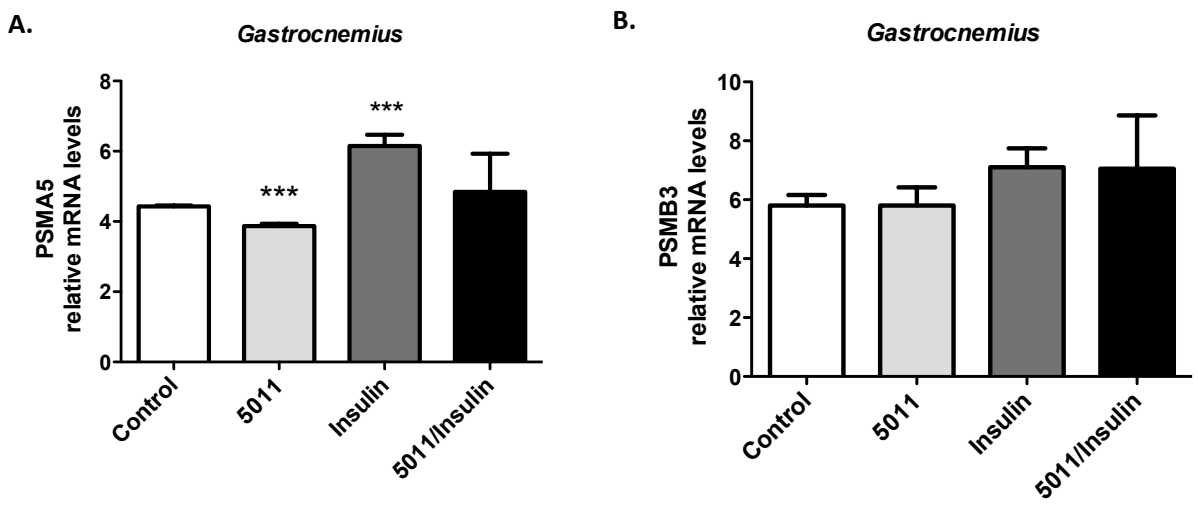


Figure 4.11 PMI 5011 Reduces Steady-State Ubiquitylation Levels in Skeletal Muscle. Whole cell extracts were isolated from skeletal muscle from control and PMI 5011 supplemented mice. The steady states of ubiquitylation were measured in (A) control versus PMI 5011 supplemented mice and in (B) insulin-stimulated control versus PMI 5011 supplemented mice. Extracts were subjected to SDS-PAGE followed by western blot analysis using anti-ubiquitin and β-actin was used as a loading control.

Based on this study, we assayed the gene expression of proteasomal subunits PSMA5 and PSMB3. PSMA5and PSMB3 showed a fold change of 2.25 and 2.27, respectively in gene expression in *gastrocnemius* skeletal muscle in diabetic rats compared to control rats (Price*, et al.,* 1996; Mitch, *et al.,* 1999). We found that KK-A^y mice supplemented with PMI 5011 lead to small, but highly significant decrease (***=$p \leq 0.001$) in PSMA5 gene expression as shown in Figure 4.12A, but had no effect on PSMB3 gene expression (Fig. 4.12B**)** in gastrocnemius skeletal muscle.



Figrue 4.12 PMI 5011 Regulates Gene Expression of PSMA5, but Not PSMB3. Gene expression for two proteasome subunits, PSMA5 (A) and PSMB3 (B) was analyzed by real-time Rt-PCR. The data are reported as the mean -/+ standard deviation (N=4/group). Statistical significance was compared to control mice. *=p≤0.05, ***=p≤0.001.

At the epicenter of the ubiquitin-proteasome system is the 26S proteasome that carries out the degradation of proteins. The 26S proteasome is a 2.5-MDa complex that is composed of around 31 different subunits that are responsible for catalyzing protein degradation. The 26S proteasome is composed of a barrel-shaped proteolytic core complex called the 20S proteasome and is capped at one or both ends by a 19S regulatory complex that recognizes ubquitinated proteins and then unfolds and translocate the ubquitinated targets into the interior of the 20S complex where they are degraded into smaller oligopeptides (Voges, *et al.,* 1999). In the 20S portion of the proteasome, there are six active sites; two sites are chymotrypsin-like, two are trypsin-like and two are caspase-like in their activity (Kisselev, , 2006). The three pairs of proteolytic sites have distinct substrate specificities. To be specific, the β5 proteolytic sites are chymotrypsin-like, the β2 sites are trypsin-like, and the β1 sites cleave after acidic residues and are

referred to as post-acidic, post-glutamate peptide hydrolase or caspase-like (Britton, *et al.,* 2009). Chymotrypsin-like proteasome activity is required for proteasome-dependent protein degradation in coordination with either one or both of the trypsin-like or caspase-like protease activity (Kisselev, *et al.,* 2006).

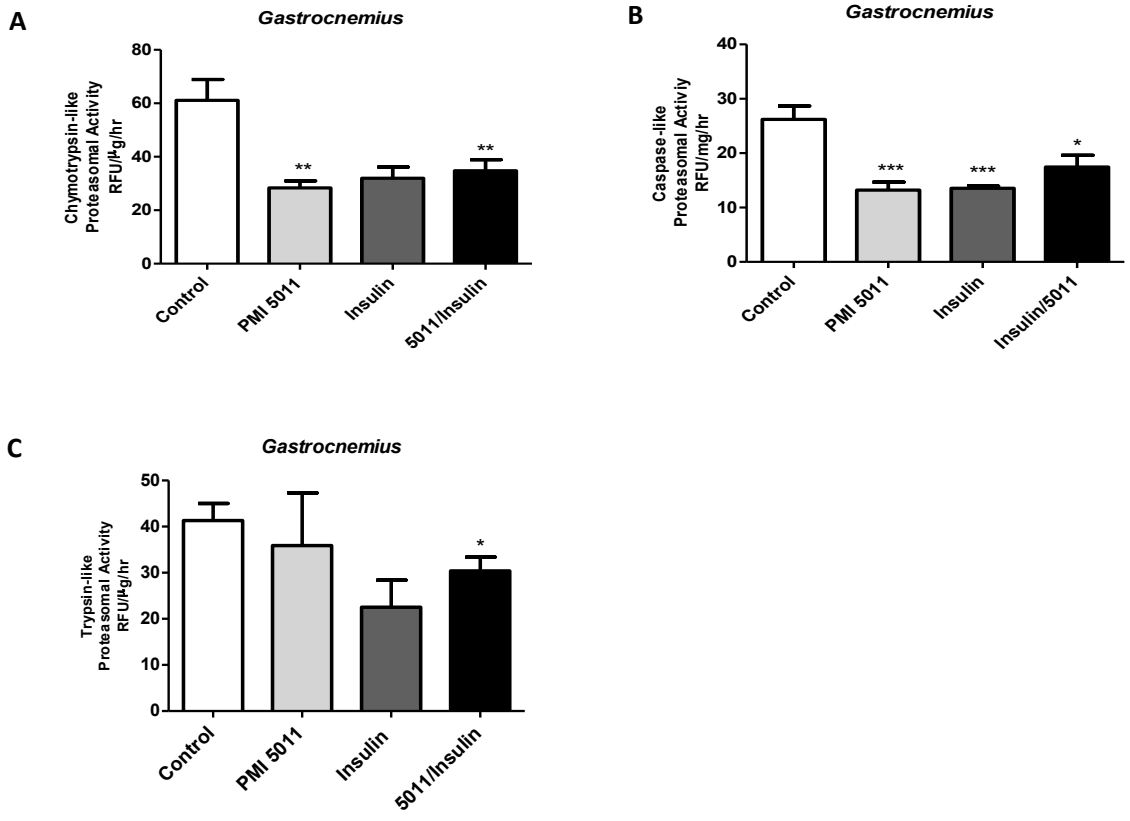


Figure 4.13. PMI 5011 Regulates Proteasomal Activity in Skeletal Muscle. Proteasomal activity for caspase-like (**A**), chymotrypsin-like (**B**) and trypsin-like (**C**) was assayed from whole cell extracts in a buffer containing MgATP to maintain the 26S proteasome structure. The data are reported as the mean -/+ standard deviation (N=4/group). Statistical significance was compared to control mice. *=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$.

We next assessed the overall effects of PMI 5011 on each of the three independent proteasomal activities in skeletal muscle. As shown in Figure 4.13, PMI

5011 substantially reduces chymotrypsin-like (A) and caspase-like (B) activity but had only slight, however significant effects on trypsin-like (C) in gastrocnemius skeletal muscle. In addition, we found that insulin-stimulation reduced all three types of activity as shown in Figure 4.13 A, B, C.

In addition to ubiquitin proteasome protein degradation, there are several non-proteasomal proteolytic pathways. In fact, the ubiquitin proteasome is incapable of breaking down complex proteins contained in myofibrils that actually constitute the bulk of proteins found in skeletal muscle (Du, *et al.,* 2004; Tiao, *et al.,* 2004; Mitch, *et al.,* 1996). Therefore, additional proteases are required to release basic proteins that make up myofibrils before the ubiquitin proteasome is able to recognize and degrade these complex proteins that make up skeletal muscle (Du, *et al.,* 2004). In order to accomplish this, the calcium-dependent proteases known as calpains are most likely required (Wing, *et al.,* 2011). It is thought that calpain activity is important in releasing components of myofibrils for degradation in the ubiquitin proteasome. In addition to the calpains, another group of proteases capable of releasing more complex myofibers are the caspases. Proapoptotic gene expression leads to activation of the caspases, which increases muscle protein degradation and muscle atrophy (Vazeille, *et al.,* 2008). Many studies have shown that the proapoptotic protease caspase-3 is required to cleave the actinomyosin and myofibril complexes of skeletal muscle proteins thereby generating actin fragments that can then be degraded by the ubiquitin proteasome system (Plant, *et al.,* 2009; Wang, *et al.,* 2010; Lee, *et al.,* 2004). However, even in the absence of apoptosis, caspase-3 is activated in rodent models of catabolic disease such as diabetes leading to cleavage of skeletal muscle actin and this caspase-3 activation has been shown to be an initial critical

steps in skeletal muscle loss (Lee, *et al.,* 2004; Du*, et al.,* 2004) much like that observed in calpain coordinated protein degradation with the ubiquitin proteasome. One last proteolytic system that also coordinately participates with ubiquitin proteasome protein degradation is the lysosomal pathway. Although our studies did not address the effect of PMI5011 on this group of proteases, the lysosomal proteases also play an important role in skeletal muscle loss associated with disease. Cathepsins are the major lysosomal proteases and it has been recognized that cathepsin L is a general marker of muscle atrophy associated with disease (Bechet, *et al.,* 2005). Cathepsin L is induced early in catabolic states and has been found to be upregulated in type 2 diabetes (Huang, *et al.,* 2003). In addition, glucocorticoid-induced muscle wasting (Dardevet, *et al.,* 1995) was associated with increased levels of cathepsin B and D mRNA. These cathepsin proteases of the lysosome likely act in concert with the ubiquitin proteasome (Baracos, *et al.,* 1995; Wing and Goldberg *,* 1995) in addition to caspases and calpains (Combaret *et al.,* 1996) or with both (Mansoor *et al.,* 1996; Taillandier*, et al.,* 1996) in coordinated degradation of skeletal muscle proteins.

In order to study the effects of PMI 5011 on non-proteasomal activity in gastrocnemius skeletal muscle we measured caspase-like, chymotrypsin-like and trypsin-like activity. We found that in addition to PMI 5011's ability to regulate all three activities of the proteasome, it also regulated non-proteasomal activity in skeletal muscle. PMI 5011 significantly regulated chymotrypsin-like, caspase-like and trypsin-like non-proteasomal activity in skeletal muscle from the insulin resistant KK-A^y mice.

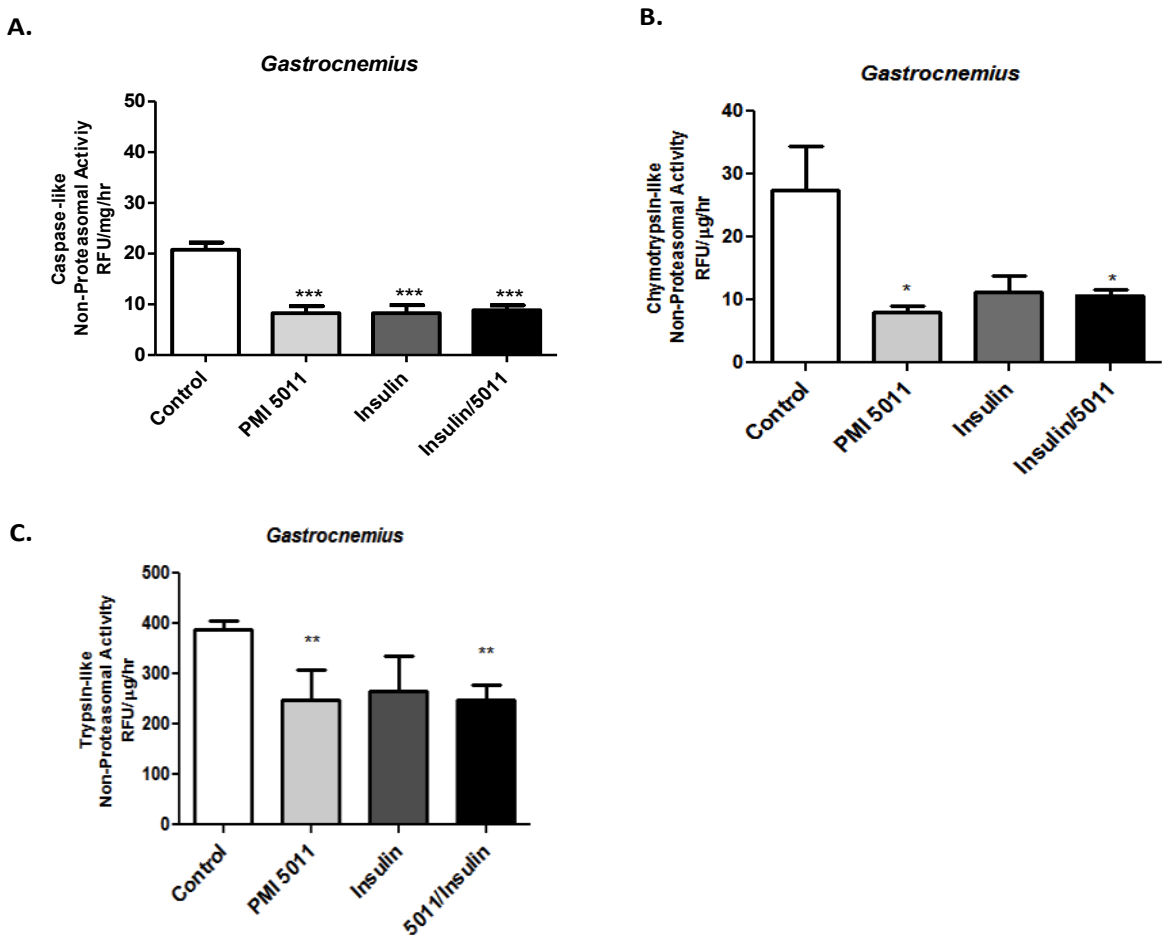


Figure 4.14 PMI 5011 Regulates Non-Proteasomal Protease Activity in Skeletal Muscle. Non-proteasomal activity for chymotrypsin-like (**A**), caspase-like (**B**) and trypsin-like (**C**) was assayed from whole cell extracts in a buffer containing MgATP to maintain the 26S proteasome structure. The data are reported as the mean -/+ standard deviation (N=4/group). Statistical significance was compared to control mice. *=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$.

As shown in Figure 4.14A, PMI 5011 alone significantly decreased non-proteasomal chymotrypsin-like activity ($p \leq 0.05$) in addition to insulin-administered animals ($p \leq 0.05$). The most dramatic effect of PMI 5011 was observed in Figure 4.14B where PMI 5011 alone significantly lowered non-proteasomal caspase-like activity ($p \leq 0.0001$) in addition to insulin-stimulated mice ($p \leq 0.0001$) as compared to control mice. Last, we found that PMI 5011 alone significantly decreased the non-proteasomal

trypsin-like activity ($p \leq 0.01$) as seen in Figure 4.14C, as well as in insulin-stimulated mice ($p \leq 0.01$) when compared to control mice. This data demonstrates that in addition to regulating all three activities of proteasomal protein degradation, PMI 5011 has significant effects on the regulation of three types of non-proteasomal activity as well.

Lastly, we performed histological H & E staining on gastrocnemius skeletal muscle to access the differences in cross-sectional areas of myofibers between control mice versus those mice supplemented with PMI 5011. In Figure 4.15A,B**,** we found that those animals supplemented with PMI 5011 had significantly larger ($p=0.02$) myofibers than control animals. Interestingly, we also found that those animals supplemented with PMI 5011 had less fat deposition as shown by the degree of white striations in control animal skeletal muscle compared to PMI 5011 supplemented mice in Figure 4.15A.

4.2. Discussion

The ethanolic extract of Russian tarragon (*Artemisia dracunculus,* L.) termed PMI 5011 was found previously to lower both serum glucose and insulin levels in KK-A^y mice, a murine model of diabetes and insulin resistance as shown by Wang, , 2011. This study also supported these findings that a diet supplemented with PMI 5011 improves glucose disposal and enhances insulin sensitivity in a mouse model of insulin resistance and diabetes. PMI 5011 has also been shown to enhance insulin signaling in skeletal muscle through increased phosphorylation of Akt by Wang, *et al.*, 2011. Because PI3K/Akt signaling regulates components of the ubiquitin proteasome system associated with muscle atrophy, we chose to evaluate the effects of PMI 5011on several components of the ubiquitin proteasome system.

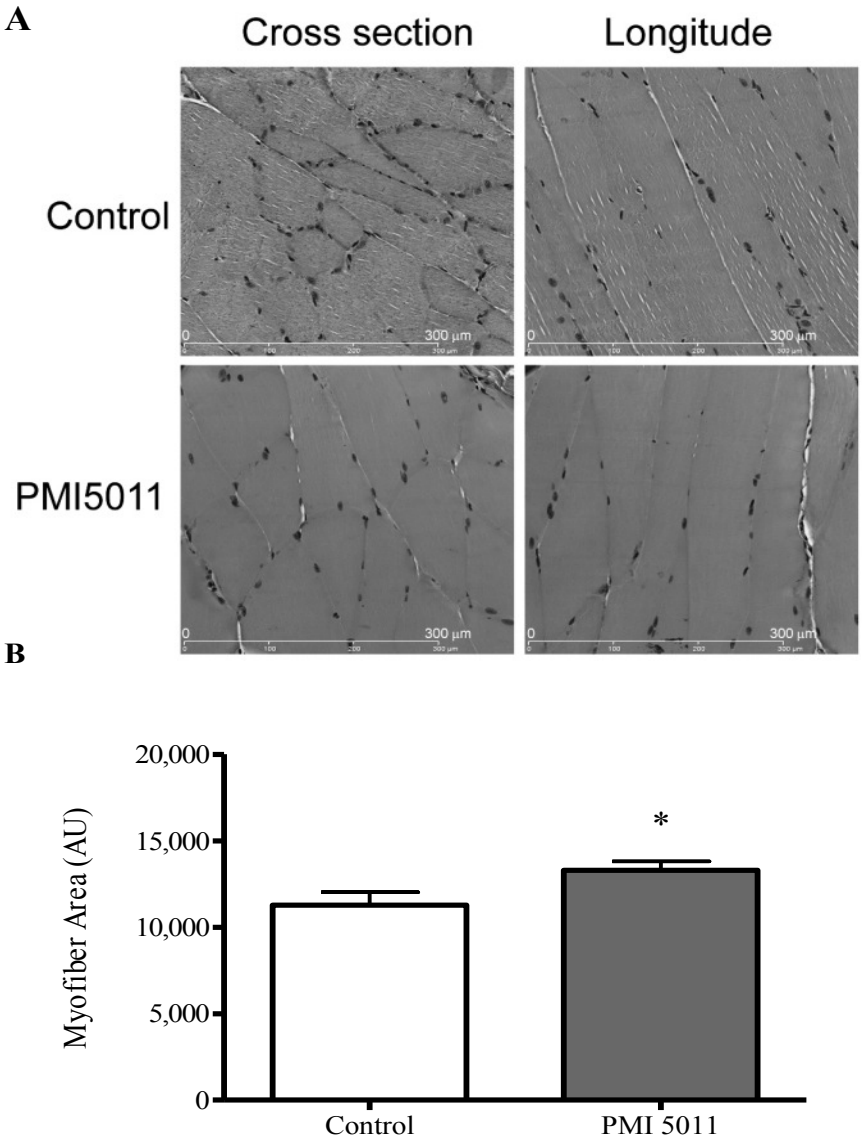


Figure 4.15 Myofiber Size is Larger in the PMI 5011 Supplemented Diet. Cross-section and Longitudinal sections of gastrocnemius skeletal muscle from control and PMI 5011 supplemented mice. (A) Myofibers were stained with hematoxylin and eosin stain. (B) Area of myofiber sizes from control and PMI 5011 supplemented mice. The data are reported as the mean -/+ standard deviation (N=4/group). Statistical significance was compared to control mice. *=$p<0.05$.

Overall, we find that PMI 5011 regulates muscle mass in the presence of insulin resistance by regulating the ubiquitin proteasome system on several levels. First, we demonstrated that PMI 5011 regulates both protein and gene expression of the two muscle-specific E3 ubiquitin ligases Atrogin-1 and MuRF-1 *in vivo* in gastrocnemius skeletal muscle of KK-A^y mice. In our most striking evidence, we demonstrate that PMI 5011 significantly regulates all three activities of both proteasomal and non-proteasomal protein degradation. We found that PMI 5011 significantly reduced both the chymotrypsin and caspase-like protease activities in both the presence as well as absence of insulin stimulation. The effects of PMI 5011 on trypsin-like proteasomal activity were only significant in the presence of insulin. However, the non-proteasomal trypsin-like activity was significantly regulated in skeletal muscle of PMI 5011 supplemented mice in both the presence and absence of insulin. Inhibition of the non-proteasomal chymotrypsin-like activity by PMI 5011 is consistent with PMI 5011 inhibition of calpain activity since the substrate used to measure chymotrypsin-like activity (Leu-Leu-Val-Tyr) is also used to assay calpain activity. PMI 5011 inhibition of calpain activity could be particularly important for degradation of the myofibrillar proteins, the main target for skeletal muscle protein degradation in muscle atrophy. MuRF-1 is the ubiquitin ligase responsible for recognizing the myofibrillar proteins once they are cleaved by the calpains (Guttman and Johnson, 1998). Therefore, our data is consistent with PMI 5011 regulation of the degradation of myofibrillar proteins at the step of calpain and ubiquitin proteasome system recognition by MuRF-1 and the proteasome itself.

We also show that the effect of PMI 5011 on the ubiquitin proteasome system is mediated through PI3K/Akt signaling pathway as evidenced by an increase in phosphorylated Akt in skeletal muscle. Activation of Akt directly inhibits FOXO transcription factors thereby, blocking upregulation of both Atrogin-1 and MuRF-1 ubiquitin ligases (Glass, 2005). Our *in vivo* studies show that the increased phosphorylation of Akt correlates with increased phosphorylation of FoxO3a, a modification that renders FoxO3a inactive as a transcription factor. As a transcription factor, FoxO3a regulates the gene expression of Atrogin-1 and MuRF-1. In addition, FoxO-dependent transcription of Atrogin-1 and MuRF-1 is required for muscle loss (Schakman, *et al.,* 2008; Zhao, *et al.,* 2007; Sandri, *et al.,* 2004). Therefore, our findings are consistent with the PMI 5011 regulation of Atrogin-1 and MuRF-1 expression via Akt inactivation of Foxo3a. downregulation of both *atrogin-1* and *MuRF-1* gene expression provides a possible link between PMI 5011 and the observed reductions of both Atrogin-1 and MuRF-1 protein expression and Akt-dependent regulation of transcriptional activity of FoxO3a. We also found that PMI 5011 regulated the gene expression of the proteasomal subunit alpha 5 (PSMA5), as well as Ube2v1 the ubiquitin-conjugating enzyme E2 variant 1 in skeletal muscle. In addition to controlling both proteasomal and non-proteasomal activities in skeletal muscle *in vivo*, we found that the steady-state levels of ubiquitylated proteins in skeletal muscle were also reduced in the PMI 5011 supplemented mice and this effect was enhanced in the presence of insulin. Lastly, we found that PMI 5011 decreased the level of Ubiquitin A52 mRNA expression levels in skeletal muscle. This data provides evidence that suggests an overall and extensive regulation of protein degradation in skeletal muscle by the botanical extract of the

perennial herb *Artemisia dracunculus* L. (PMI 5011) in catabolic conditions such as insulin resistance and type 2 diabetes. This broad regulation of activities involved in protein degradation in skeletal muscle *in vivo* by PMI 5011 has important implications for its therapeutic possibilities in atrophy associated with catabolic diseases.

CHAPTER 5: SUMMARY AND CONCLUSIONS

5.1 Summary

The botanical extract, PMI 5011 lowers blood glucose and insulin levels and enhances insulin signaling in skeletal muscle leading to preserved muscle mass through regulation of protein degradation. Given the important role of skeletal muscle in glucose disposal and protein storage in the body, the effect of PMI5011 on skeletal muscle protein degradation may have implications for the treatment of type 2 diabetes.

Overall, we find that PMI 5011 regulates muscle mass in the presence of insulin resistance by regulating the ubiquitin proteasome system on several levels. In the overall process, ubiquitylation is carried out by a set of three enzymes, E1, E2 and E3. Ubiquitin is first activated by ubiquitin-activating enzyme E1, in an ATP-dependent manner. Next, the ubiquitin molecule is passed on to the second enzyme of the complex, E2 ubiquitin-conjugating enzyme. Next, it is recognized by the final enzyme, E3, the ubiquitin protein ligase that binds the target substrate and labels it with ubiquitin. The process can be repeated until a short chain is formed, with three or more ubiquitin molecules targeting the protein to the proteasome (Wing, 2005). We have demonstrated through our experiments that PMI 5011 regulates almost every step of this process. As depicted in the Figure 5.1, below, we show that PMI 5011 a) regulates the level of ubiquitin A52, a form of ubiquitin that is fused with ribosomal protein L40 and may aid in assembling the ribosome (Baker and Board, 1991) , b) regulates the activity of Ube2v1, a protein that shares sequence and structure similarity to E2 enzymes and is thought to interact with E2 enzymes to provide a higher degree of enzyme specificity (Sancho, *et al.,* 1998), c)

regulates the two muscle-specific E3 ubiquitin ligases, Atrogin-1 and MuRF-1, d) regulates steady-state levels of ubiquitylation, e) regulates the proteasomal subunit PSMA5 gene expression and f) regulates all three activities of the proteasome.

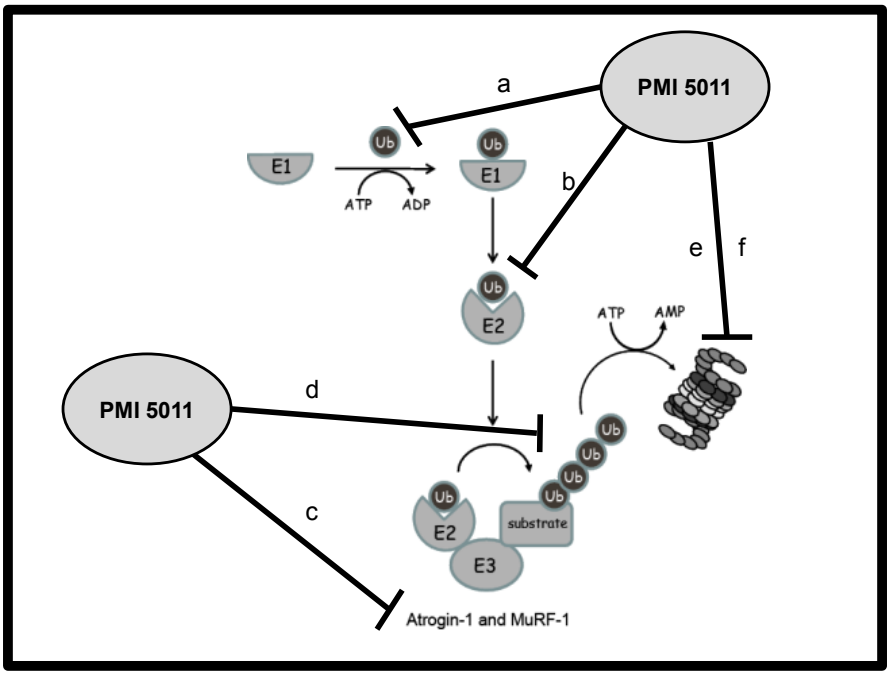


Figure 5.1. PMI 5011 Regulates Several Levels of Ubiquitin Proteasomal Protein Degradation.

In addition to overall regulation of the many facets of the ubiquitin proteasome system, we provide further evidence regarding the mechanism by which PMI 5011 regulates the expression of Atrogin-1 and MuRF-1. We found *in vitro* and *in vivo* that PMI 5011 enhanced phosphorylation of Akt. Our *in vivo* studies show that the increased phosphorylation of Akt correlates with increased phosphorylation of FoxO3a, a modification that renders FoxO3a inactive as a transcription factor. As a transcription factor, FoxO3a regulates the gene expression of Atrogin-1 and MuRF-1 and FoxO-

dependent transcription of Atrogin-1 and MuRF-1 is required for muscle loss (Schakman, *et al.,* 2008; Zhao, *et al.,* 2007; Sandri, *et al.,* 2004). Therefore, our findings are consistent with the PMI 5011 regulation of Atrogin-1 and MuRF-1 expression via Akt inactivation of Foxo3a. Additionally, PMI 5011 regulated the steady-state levels of ubiquitylated proteins. Bachmair*, et al.,* 1989 hypothesized that ubiquitylation of a protein requires two steps: recognition of the targeted protein by a ubiquitin ligase followed by attachment of ubiquitin to the protein. The PMI5011-mediated decrease in ubiquitylation levels may be a consequence of the reduced expression of the two muscle-specific ubiquitin ligases Atrogin-1 and MuRF-1 and a corresponding reduction in the activity of each ligase. However, we cannot rule out the possibility that PMI5011 is affecting recognition of a ubiquitin-modified protein at the proteasome.

Perhaps our most striking evidence that PMI 5011 inhibits skeletal muscle loss is by inhibiting the actions of all three protease activities of the proteasome *in vivo*. This project showed that the beneficial effect of PMI 5011 on insulin signaling extends to the regulation of ubiquitin-proteasome activity in addition to non-proteasomal activity in skeletal muscle, indicating that PMI 5011 is a potent inhibitor of overall skeletal muscle protein turnover *in vivo*. All three activities of the proteasome including caspase-like, chymotrypsin-like, and trypsin-like activities in skeletal muscle were significantly reduced by PMI 5011 while the related activities of non-proteasomal proteases in skeletal muscle were also significantly reduced by PMI5011. Lastly, we observed in histological sections of Hematoxylin and Eosin (H & E) stained gastrocnemius muscle tissue that PMI 5011 treated animals had larger overall myofiber size compared to KK-A^y controls, providing morphological evidence of the beneficial effects of PMI 5011 in preserving

muscle mass in the presence of insulin resistance. This extensive inhibition and regulation of protein degradation in skeletal muscle is a very powerful means by which PMI 5011 could potentially protect skeletal muscle mass in catabolic diseases such as type 2 diabetes. In summary, these studies demonstrate that PMI 5011 regulates protein degradation in insulin resistant states *in vitro* and *in vivo*. PMI 5011 may therefore be a therapeutic target for the conservation of muscle mass in catabolic conditions such as type 2 diabetes by directly targeting several components of the ubiquitin proteasome system.

5.2 Conclusions

In conclusion, there is a dynamic balance between protein synthesis and degradation that regulates muscle mass. Insulin resistance in type 2 diabetes is associated with impaired glucose and protein metabolism of the skeletal muscle. The impaired insulin signaling in skeletal muscle affects muscle mass by tipping the balance from skeletal muscle protein synthesis and degradation toward degradation in a process that is primarily regulated by the ubiquitin-proteasome system. As the major site of protein breakdown the ubiquitin proteasome has been shown to be significantly upregulated in muscle atrophy associated with insulin resistance (Park, *et al.,* 2009; Wang, *et al.,* 2006). Studies have shown that an extensively characterized ethanol extract of *Artemisia dracunculus* L (Russian Tarragon), termed PMI 5011, enhances insulin signaling in human primary skeletal muscle cells and in a rodent model of insulin resistance (Wang, *et al.,* 2008 and Wang*, et al.,* 2011) and this enhanced insulin signaling was associated with an increase in protein levels in skeletal muscle (Wang*, et al.,* 2011).

This data provides evidence that suggests an overall and extensive regulation of protein degradation in skeletal muscle by the botanical extract of the perennial herb *Artemisia dracunculus* L. (PMI 5011) in catabolic conditions such as insulin resistance and type 2 diabetes. One of the most important findings of this study was that PMI 5011 significantly regulated all three protease activities of the proteasome in addition to the activity of three related non-proteasomal classes of proteases. Chymotrypsin-like proteasome activity is required for overall ubiquitin-proteasome protein degradation in coordination with either one or both of the trypsin-like or caspase-like proteasome activities (Kisselev, *et al.,* 2006). In fact, the proteasome is incapable of breaking down complex proteins contained in myofibrils that actually constitute the bulk of proteins found in skeletal muscle (Du, *et al.,* 2004; Tiao, *et al.,* 2004; Mitch, *et al.,* 1996). Therefore, additional proteases are required to release basic proteins that make up myofibrils before the proteasome is able to recognize and degrade these complex proteins that make up skeletal muscle (Du, *et al.,* 2004). In order to accomplish this, the calcium-dependent proteases known as calpains are most likely required (Wing, *et al.,* 2011). Of the 14 genes encoding calpain proteases, 7 different calpains are expressed in skeletal muscle and evidence has been provided that links calpain activation with increased ubiquitin proteasome activity. It is thought that calpain activity is important in releasing components of myofibrils for degradation in the ubiquitin proteasome. *CAPN3* knock-out mice show an abnormal accumulation of high molecular weight ubiquitin-protein conjugates in muscle during reloading after disuse and this data that suggests an important role for CAPN3 in ubiquitin-proteasome system degradation of products of CAPN3 cleavage (Kramerova, *et al.,* 2005). Inhibition of the non-proteasome

chymotrypsin-like activity by PMI5011 is consistent with PMI5011 inhibition of calpain activity since the substrate used to measure chymotrypsin-like activity (Leu-Leu-Val-Tyr) is also used to assay calpain activity (Guttman and Johnson, 1998). PMI5011 inhibition of calpain activity could be particularly important for degradation of the myofibrillar proteins, the main target for skeletal muscle protein degradation in muscle atrophy. MuRF-1 is the ubiquitin ligase responsible for recognizing the myofibrillar proteins once they are cleaved by the calpains (Cohen, *et al.,* 2009). Therefore, our data is consistent with PMI5011 regulation of the degradation of myofibrillar proteins at the step of calpain and ubiquitin proteasome system recognition by MuRF-1 and the proteasome itself.

In addition to the calpains, another group of proteases capable of releasing more complex myofibers are the caspases. Many studies have shown that the proapoptotic protease caspase-3 is required to cleave the actinomyosin and myofibril complexes of skeletal muscle proteins thereby generating actin fragments that can then be degraded by the ubiquitin proteasome system (Plant, *et al.,* 2009; Wang, *et al.,* 2010; Lee, *et al.,* 2004). Studies have shown that caspase-3 is activated in rodent models of catabolic disease such as diabetes. The activated caspase-3 cleaves skeletal muscle actin and this caspase-3 activation has been shown to be an initial critical steps in skeletal muscle loss (Lee, *et al.,* 2004; Du*, et al.,* 2004), much like that observed in calpain coordinated protein degradation with the ubiquitin proteasome. One last proteolytic system that also coordinately participates with ubiquitin proteasome protein degradation is the lysosomal pathway. Although our studies did not address the effect of PMI5011 on this group of proteases, the lysosomal proteases also plays an important role in skeletal muscle loss

associated with disease. Cathepsins are the major lysosomal proteases and it has been recognized that cathepsin L is a general marker of muscle atrophy associated with disease (Bechet, *et al.,* 2005). Cathepsin L is induced early in catabolic states and has been found to be upregulated in type 2 diabetes (Huang, *et al.,* 2003). In addition, glucocorticoid-induced muscle wasting (Dardevet, *et al.,* 1995) was associated with increased levels of cathepsin B and D mRNA. These cathepsin proteases of the lysosome are likely act in association with the ubiquitin proteasome system (Baracos, *et al.,* 1995; Wing and Goldberg*, et al.,* 1995) in addition to caspases and calpains (Combaret *et al.,* 1996) or with both (Mansoor *et al.,* 1996; Taillandier*, et al.,* 1996) in coordinated degradation of skeletal muscle proteins.

Due to the importance of these additionally coordinated proteolytic systems with ubiquitin proteasome protein degradation and the evidence provided by this study that PMI 5011 regulates non-proteasomal protein degradation, future studies with PMI 5011 should therefore seek to determine the effects of PMI 5011 on muscle-specific caspase, calpain and cathepsin expression in association with muscle atrophy provided in these models.

5.3 Future Studies

Studies to determine the level of CAPN3 expression in skeletal muscle of mice supplemented with PMI 5011 could provide further insight into the effects of PMI 5011 on the coordinated regulation of calcium-dependent proteolysis and the ubiquitin proteasome system. A possible role for the muscle-specific lysosomal Cathepsin L in coordination with the ubiquitin-proteasome system (Huang, *et al.,* 2003), would provide additional information on the overarching capabilities of PMI 5011 to regulate protein

degradation as will examination of the effect of PMI5011 on caspase activity in skeletal muscle.

Another link to be made in future studies is through the PI3K/Akt signaling pathway that regulates both Atrogin-1 and MuRF-1 by directly inhibiting the FoxO transcription factors known to induce both *atrogin-1* and *MuRF-1* gene expression. In Akt-dependent phosphorylation of FoxO1 or FoxO3a, both are excluded from the nucleus where these transcription factors can no longer upregulate the transcription of *atrogin-1* or *MuRF-1* gene expression (Sandri, *et al.,* 2004; Stitt, *et al.,* 2004 and Glass, 2010). To further understand the role of PMI 5011 in affecting FoxO3a-dependent regulation of these two muscle-specific ligases, FoxO3a localization should be determined by cellular fractionation followed by western blot analysis of the cytoplasmic and nuclear fractions and by *in situ* immunolocalization. This will help determine if FoxO3a is indeed being downregulated by its exclusion from the nucleus due to PMI5011.

Previous studies associated increased protein content of skeletal muscle with PMI 5011-mediated changes in insulin sensitivity (Wang, *et al.,* 2011). We find that the increase in protein content observed by Wang, *et al.,* 2011, is associated with PMI 5011 inhibition of ubiquitylation as well as proteasomal and non-proteasomal activity in skeletal muscle. However, we did not focus our efforts on the effects of PMI 5011 on protein synthesis. Insulin is a potent anabolic hormone that not only regulates protein degradation but also regulates protein synthesis by activating the mTOR pathway (Miyazaki and Esser, 2009). It is therefore, feasible that PMI 5011's insulin stimulating activity could also play a role in protein synthesis through insulin-stimulated mTOR signaling. Future studies on PMI 5011 in skeletal muscle could shift towards a focus on

its effects on protein synthesis by elucidating its role in signaling pathways such as the mTOR signaling pathway. Additionally, the translation initiation factor eIF3f is a downstream target of Atrogin-1 (Csibi, *et al.,* 2008; Csibi, *et al.*, 2009; Lagirand-Cantaloube, *et al.,* 2008). It is possible that the observed downregulation of Atrogin-1 expression by PMI 5011 could be linked to increases in protein synthesis by inhibiting the degradation of the initiation factor eIF3f. In addition to enhanced mTOR signaling, this data would provide additional evidence of PMI 5011's effects on protein synthesis. PMI 5011's possible role in protein synthesis would provide additional insight into its complete contribution to overall conservation of skeletal muscle mass.

Finally, the goal in biomedical research of using medicinal plants as a source of therapeutic agents is to target the isolation of the bioactive compounds for synthesis. Future studies should also include bioassay guided fractionation studies to determine the active compounds responsible for the regulation of protein degradation and muscle mass conservation in catabolic diseases. These experiments would initially focus on regulation of proteasome activity by subfractions of PMI 5011, including the 6-emethoxycapillarisin and 2', 4' dihydroxy-4-methoxydihydrochalcone subfractions previously identified as having anti-hyperglycemic effects (Govorko, *et al.*, 2007).

To conclude, natural drugs from traditional medicines are again gaining popularity due to fewer side effects, reduced cost, and increased patient use. Non-timber forestry products provide vital plants or plant parts to be utilized for drug discovery for the treatment of many important diseases and associated symptoms such as the muscle atrophy caused by insulin resistance and type 2 diabetes. In addition, and important to this author, non-timber forestry products contribute to sustainable forest management,

conservation, and to both economical and developmental objectives (Panayotou and Ashton, 1992). These non-timber forestry products provide dual benefits to both biomedical research and to the renewable natural resource organizations and forests of our Country. A proposed model of PMI 5011's actions and testable hypotheses related to future studies is illustrated in Figure 5.2 below.

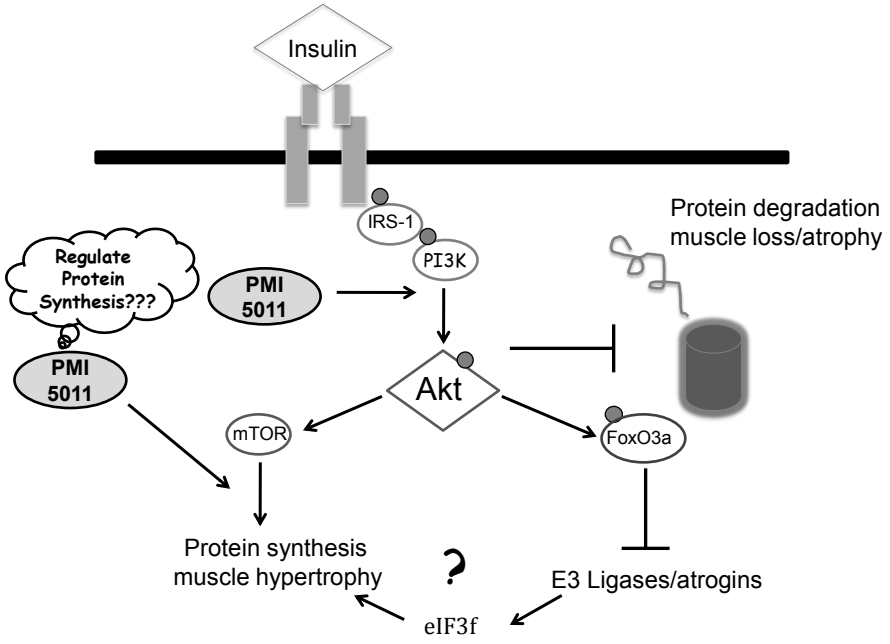


Figure 5.2 Proposed Model of PMI 5011 Regulation of Muscle Atrophy in Type 2 Diabetes.

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